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Antifungal activity of bacitracin and its interaction with metals

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Abstract. Bacitracin was more growth-inhibitory to *Neurospora crassa* on a minim magnesium medium than on a normal magnesium medium. Both magnesium and mangane were able to counteract the growth inhibition. The antifungal activity of bacitracin w potentiated by zinc. Potassium could not counteract the growth inhibition by this antibiot The mycelial magnesium levels were low in bacitracin-inhibited cultures.

Keywords. *Neurospora crassa*; bacitracin; peptide antibiotics.

Introduction

Metals like zinc, nickel, manganese and cobalt chelate with the antibiot bacitracin (Anker *et al.*, 1948; Gross *et al.*, 1954; Weinberg, 1958; Garbutt *et a* 1961). The antibacterial activity of bacitracin was enhanced by cadmium and zi both singly and in combination and occasionally by manganese (Adler and Snok 1962). It was also reported that chelation with copper inactivated bacitrac (Sharp *et al.*, 1949). These studies indicated that chelation with metals bacitracin was related to its anti-bacterial activity. The primary mode of action bacitracin is probably by the impairment of permeability of the membranes sensitive cells, resulting in the leakage of potassium ions (Hancock and Fitz-Jame 1964). This paper reports the antifungal activity of bacitracin and the effect metals on it.

Methods

Organism, media and growth conditions

A wild-strain of *Neurospora crassa* Em5297a maintained by weekly subcultures 2% agar slants, was grown in 50 ml Corning conical flasks containing 10 ml of ba medium (Sivarama Sastry *et al.*, 1962). Where necessary, bacitracin was asepticа added to the medium before inoculation with spores. The organism was allow to grow at 30 ± 1°C for 72 h and the dry weights of the mycelial pads were obtain after drying them at 70-80°C for 8 h.

Estimation of magnesium and bacitracin

The mycelial magnesium content was determined by a microbiological pr
(Sivarama Sastry *et al.*, 1963). Bacitracin was estimated by Pauly's diazo
(Block *et al.*, 1966) in order to determine the antibiotic either bound to the
or taken up by the organism.

Chemicals

Bacitracin (Calbiochem) was a gift sample from Professor L. K. Ramach
Analytical grade sulphate salts of magnesium, manganese and zinc were en
in these studies.

Results

Effect of bacitracin on the growth of N. crassa

The antifungal activity of bacitracin tested with *N. crassa* is depicted in fi
The growth inhibition by the antibiotic was examined using two types of

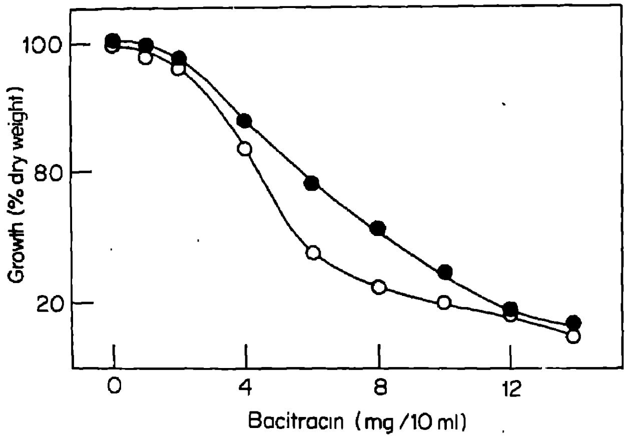

Figure 1. The effect of bacitracin on the growth of *N. crassa* Em5297a. Toxicity on no
magnesium medium(●); toxicity on minimal magnesium medium (O).

(i) a minimal magnesium medium (50 μg Mg^{2+}/10 ml) and (ii) a r
magnesium medium (500 μg Mg^{2+}/10 ml) (Sivarama Sastry *et al.*, 1962). Th
biotic appeared to be more inhibitory on the minimal magnesium m
requiring only 5 mg/10 ml to produce 50% inhibition of growth as compared
mg/10 ml to produce the same inhibition on the latter medium.

Effect of metals on the antifungal activity of bacitracin in N. crassa on m magnesium medium

The results presented in figure 2 indicate that both excess magnesiu
manganese could counteract the toxicity of bacitracin. Magnesium was a
protect the organism at a molar ratio of bacitracin to magnesium of 1:2, wl
the ratio for manganese was 20:1. (to obtain 100% growth restoration, only

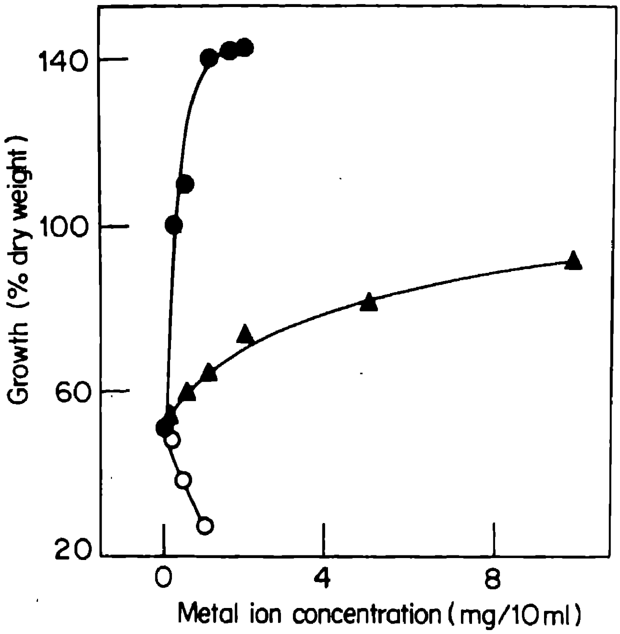

Figure 2. The effect of metals on the growth inhibition of *N. crassa* Em5297a by bacitracin on minimal magnesium/zinc medium. manganese (●), zinc (O), magnesium (▲).

manganese/10 ml was required to counteract the growth inhibition by 5 mg of bacitracin). This observation highlighted that manganese was much more efficient in reversing the growth inhibition as compared to magnesium. On the other hand, zinc could not alleviate the growth inhibition but also potentiated the antifungal activity of bacitracin. This metal, at a concentration of 1000 μg/10 ml, further reduced the growth of the organism (roughly 20% growth) in the presence of bacitracin (5 mg/10 ml medium).

Table 1. The uptake of magnesium and its effect on bacitracin content of mycelia

Bacitracin (mg/10 ml medium)	Mg^{2+} or Mn^{2+} (μg/10 ml medium)	Uptake (mg/100 mg dry wt)	
		Mg^{2+}	Bacitracin
Nil	Nil	0.085	Nil
5.0	Nil	0.040	17.0
5.0	2000 Mg^{2+}	0.080	9.1
5.0	250 Mn^{2+}	—	8.3

The organism was grown for 72 h on minimal magnesium medium. Magnesium or manganese were added to the medium where necessary along with bacitracin.

Effect of bacitracin on mycelial magnesium uptake

Magnesium was antagonistic to the action of bacitracin. The content of this metal in the antibiotic-inhibited mycelia was reduced to nearly 52%. However, the mycelial magnesium content was restored to the normal level when the inhibition was relieved by this divalent cation (table 1).

The results presented in table 1 also indicate that nearly 72% of the bacitracin added to the medium was either bound to the organism or entered the cells. But such a binding or entry of the antibiotic was drastically lowered (50% of that of toxic cultures) in the presence of excess magnesium or manganese.

Discussion

The antifungal activity of peptide antibiotics has been reported by Chalmers (1974) and Al-Saqur (1975). Bacitracin is toxic to *N. crassa*; a 50% growth inhibition being produced at a concentration of 5 mg/10 ml on a minimal magnesium medium. The inhibitory concentration obtained in this study is in close agreement with the value of Al-Saqur (1975) who reported that about 0.5 mg bacitracin/ml could inhibit the growth of *N. crassa* by 55%. The present studies also prove that more bacitracin is required to produce the same inhibition of growth on a normal magnesium medium. This antagonistic relationship between bacitracin and magnesium is more evident on a minimal magnesium medium. A comparison was made by Haavik (1976) between the toxicities of certain divalent metals and bacitracin in bacteria, where both types of toxicities were counteracted by excess magnesium. Such an antagonism between magnesium and other toxic metals like cobalt, nickel, etc. is also well-documented in *N. crassa* (Sivarama Sastry *et al.*, 1962; Venkateswerlu and Sivarama Sastry, 1973).

Divalent metals like zinc, cadmium and manganese were reported to enhance the antibacterial activity of bacitracin (Adler and Snoke, 1962). The present studies indicated that zinc enhanced the antifungal activity of bacitracin like its antibacterial activity. This metal ion is known to produce a conditioned deficiency of magnesium in fungi (Adiga *et al.*, 1961; Sivarama Sastry *et al.*, 1962); obviously the increased toxicity of bacitracin to *N. crassa* in the presence of zinc was probably due to increased magnesium deficiency. Excess supplementation of phosphate in the medium was found to be ineffective either in the reversal or in the enhancement of the toxicity (results not presented). It was confirmed that in the presence of relatively high but non-toxic amounts of manganese or cobalt, small amounts of bacitracin significantly inhibited the growth of *Bacillus leicheniformis* (Haavik, 1976). Earlier Adler and Snoke (1962) observed that manganese also enhanced the potency of bacitracin. However, the results of the present investigation are not in complete conformity with the above observation, since, the antifungal activity of this antibiotic is enhanced by zinc but not by manganese; on the other hand manganese behaved like magnesium in antagonizing the toxicity. The mechanism of reversal by manganese of the antibiotic toxicity in this mold is not clear at present. One possibility is that it may be substituting for magnesium as in many other cases.

Hancock and Fitz-James (1964) reported that bacitracin interferes with cell-membrane permeability resulting in the leakage of potassium ions. However, in preliminary studies no such interference was found to occur since excess potassium (checked upto 4000 µg K^+/10 ml) supplementation could not protect the organism from the bacitracin toxicity.

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Immobilization and properties of β- D-galactosidase from *Lactobacillus bulgaricus*

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Abstract β-D-galactosidase (EC 3.2.1.23) from *Lactobacillus bulgaricus* (1373) was immobilized by entrapment in a polyacrylamide gel lattice. The enzymatic properties of the immobilized β-galactosidase were compared with those of the native enzyme. The temperature and pH optima were not affected by the immobilization. After entrapment of the enzyme no significant change was observed in its thermostability. The pH stability of the immobilized enzyme was higher than that of the native enzyme on the acidic side. The K_m values for the immobilized and native β-galactosidase with both lactose and o-nitrophenyl-β-D-galactoside as substrates were comparable. The immobilized enzyme could be repeatedly used 12 times without any loss of activity. No loss in the activity of the immobilized β-galactosidase was found after its storage for 30 days at 4°C and for 20 days at 25°C.

Keywords Immobilized β-galactosidase; *Lactobacillus bulgaricus*; acrylamide; gel entrapment; kinetic properties.

Introduction

Enzymatic hydrolysis of lactose in milk and milk products by β-D-galactosidase alleviates the problem of lactose intolerance, disposal of whey, and crystallization of lactose in different food products (Kretchmer, 1972; Makkar and Dutta, 1979a). A number of β-galactosidases have been studied for their potential applications in the hydrolysis of lactose (Sharp *et al.*, 1969; Wendorff *et al.*, 1971; Woychik and Wondolowski, 1972, 1973; Olson and Stanley, 1973; Dahlavist *et al.*, 1973; Husted *et al.*, 1973; Wondolowski and Woychik, 1974; Weetall *et al.*, 1974). The enzymes were essentially of yeast or fungal sources, although some reports were available on bacterial-galactosidases (Sharp *et al.*, 1969; Hustad *et al.*, 1973; Wondolowski and Woychik, 1974). The yeast or fungal β-galactosidases have optimum pH of around 4.5 which cannot be efficiently used for the hydrolysis of skim milk and sweet whey whose pH varies from 6.2 to 7.0. One exception is the yeast *Saccharomyces lactis* β-galactosidase with optimum pH 7.0 which is thermo-labile above 30°C (Woychick *et al.*, 1974). So it becomes imperative to examine other sources, preferably bacterial because of their likely higher pH optima. Studies for the evaluation of immobilized β-galactosidase of bacterial origin are scanty (Sharp *et al.*, 1969; Hustad *et al.*, 1973; Wondolowski and Woychik, 1974) and are primarily limited to *Escherichia coli.* The matrices used in these studies are porous cellulose (Sharp *et al.*, 1969), porous glass beads (Wondolowski and Woychik, 1974), polyisocyanate polymers (Hustard *et al.*, 1973) etc. Rao and Dutta (1978) screened a large number of micro-organisms to select a potential source of β-galactosidase for food applications. They reported *Lactobacillus bulgaricus* (1373) to be one of the most promising isolates. With a view to use β-galactosidase from this bacterial source for the hydrolysis of lactose, it was immobilized by entrapping in an acrylamide gel lattice. The advantages of this method over the other methods are that it offers a convenient procedure for the immobilization of a larger amount of enzyme and the distribution of the enzyme in the carrier is uniform (Ohmiya *et al.*, 1974). In addition, it is inexpensive and resistant to biological attack (Whitesides *et al.*, 1975). This paper reports the conditions of entrapping β-galactosidase from *Lactobacillus bulgaricus* (1373) in acrylamide gel, comparison of the properties of the immobilized and native forms of β-galactosidase and stability studies of the immobilized enzyme after repeated use and storage.

Materials and methods

Acrylamide monomer was purchased from Biochemical Unit, V.P. Chest Institute, Delhi; N, N′-methylene-bisacrylamide, N.N, N′, N′-tetramethyl-ethyenediamine (TEMED), o-nitrophenyl-β-D-galactopyranoside (nitrophenyl galactopyranoside) and glucose kit were purchased from Sigma Chemical Company, St. Louis, Missouri, USA and potassium persulfate and lactose from British Drug House (India). The culture of *L. bulgaricus* (1373) was obtained from the culture collection of Natural Dairy Research Institute, Karnal.

Production of β-galactosidase II

Whey (about 1.5 litre) was deproteinized by heating at 95°C for 5 min at pH 4.5 and filtered to remove the coagulated proteins. It was supplemented with 0.5% (w/v) proteose peptone, pH adjusted to 8.5 and steamed for 15 min in an autoclave. One litre of the suplemented whey medium was cooled to about 30°C and then inoculated with 10 ml of the active culture of *L. bulgaricus.* It was incubated at 42°C for 24 h. Maximum production of the enzyme was obtained under the above conditions (to be published elsewhere). At the end of the incubation period, cells were harvested by centrifugation at 5,000 *g* for 15 min. The cell pellet was washed twice with phosphate buffer (pH 6.5, 0.05 M), resuspended in the same buffer and disintegrated using Vibronics ultrasonic processor at 80 mA for 30 min at 0-4°. The cell debris was removed by centrifugation at 20,000 g for 20 min at 4°C. The cell-free extract was brought to 40% saturation with solid ammonium sulphate. It was centrifuged and the precipitate obtained was discarded. The supernatant was brought to 60% saturation by a further addition of ammonium sulphate and the resulting precipitate was collected by centrifugation. It was dissolved in phosphate buffer (pH 6.5, 0.05 M) and dialyzed against double glass distilled water for 24 h at 4°C. This enzyme preparation purified 7 fold, was used in all further experiments.

Standard preparation of immobilized β-galactosidase

To 2.0 g of acrylamide plus 88 mg bisacrylamide, 8 ml of a solution of β-galactosidase (0.375 mg protein/ml) was added and the solution maintained at 4°C. One ml each of TEMED (5%) and potassium persulphate (5%) were added and the mixture kept at 0-4°C to avoid thermal inactivation of the enzyme during polymerization, which was complete in 5-10 min. The resulting gel was homogenized in phosphate buffer (pH 6.5, 0.5 M) with intermittent cooling. The gel particles were washed twice with the same buffer and suspended in 0.05 M phosphate buffer (pH 6.5). This preparation was used for characterizing the immobilized β-galactosidase.

Assay of native galactosidase

With lactose as a substrate, enzyme activity was determined by the procedure of Dahlqvist (1964) with slight modifications. One ml of 5% lactose (0.139 M) solution in phosphate buffer (pH 6.5, 0.05M) was mixed with the enzyme solution in a total volume of 2.0 ml and incubated at 42°C. The reaction was stopped after 1 h by heating the tubes in a boiling water bath for 5 min. One ml of the reaction mixture was deproteinized by adding 1.0 ml of 5% $ZnSO_4 7H_2O$ and 1.0 ml of 4.5% $Ba(OH)_2$ and filtered. Glucose produced was estimated in 1 ml of the filtrate by the glucose-oxidase procedure Jasewicz and Wasserman, 1961.

With nitrophenylgalactopyranoside as substrate, *o*-nitrophenol released at 42°C in 10 min was determined following the procedure of Wendorff *et al.* (1971).

Assay of immobilized β-*galactosidase*

With both lactose and nitrophenylgalactopyranoside as substrate the assay method was essentially the same as for the native enzyme, except that the immobilized enzyme suspension was used instead of free enzyme and that the reaction mixture was incubated with shaking (100 rpm). A unit of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of *o*-nitrophenol per min at 42°C.

Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Results

Conditions of immobilization

Effect of concentrations of acrylamide and bisacrylamide: Table 1 shows the effect of varying the concentrations of acrylamide and bisacrylamide on the activities of immobilized β-galactosidase. The highest activity yield of immobilized enzyme was obtained when 2 g of acrylamide monomer and 88 mg of bisacrylamide were used in the reaction mixture of volume 8 ml. The activity yield of immobilized enzyme decreased with further increase in the concentration of acrylamide.

Effect of β-*galactosidase concentration:* The effect of the concentration of β-galactosidase on immobilization was investigated (table 2). The activity yield of the immobilized enzyme increased with increase in the concentration of the

Table 1. Effect of different concentrations of acrylamide and bisacrylamide on the activity of immobilized β-galactosidase.

		Immobilized β-Galactosidase	
Acrylamide (g)	BIS (mg)	Total activity* (units)	Activity yield* (%)
1.0	80	112	18
1.5	80	138	22
2.0	80	174	28
2.5	80	124	20
3.0	80	106	20
2.0	40	124	20
2.0	80	174	20
2.0	88	188	30
2.0	100	188	30

Native β-galactosidase added (3 mg protein, 624 units).
* With nitrophenylgalactopyranoside substrate

Table 2. Effect of concentration of β-galactosidase on the activity of immobilized β-galactosidase.

Native β-Galactosidase used		Immobilized β-Galactosidase	
Amount (mg)	Total activity* (units)	Total activity* (units)	Activity yield (%)
1.0	208	46	22
2.0	416	112	27
3.0	624	200	31
4.0	932	208	25

Immobilization was carried out under standard conditions described in the text except for the concentrations of β-galactosidase.
* With nitrophenylgalactopyranoside substrate.

enzyme and was found to be maximum when 3.0 mg of the native enzyme w added in the reaction mixture (8.0 ml). Above this concentration, the activi yield was found to decrease.

Properties of immobilized β-galactosidase

Effect of shaking: The effect of shaking the reaction mixture at different spee on the activity of immobilized enzyme was investigated to see if the reaction w diffusion-controlled. The activity of the immobilized enzyme was lower when th reaction was catalysed without shaking the reaction mixture than when the reaction mixture was stirred at 100 rpm or 200 rpm. The activity reached its maximum when the stirring speed of the reaction mixtures was maintained at 50rpm or more, showing that the diffusional limitations were eliminated at this speed.

Effect of pH: Maximum activity of the immobilized enzyme with both lactose and nitrophenylgalactopyranoside as substrates was observed at pH 6.5. The native enzyme was also maximally active at pH 6.5.

Effect of temperature: The effect of temperature on the hydrolysis of lactose and nitrophenylgalactopyranoside by the immobilized β-galactosidase was compared with that of native β-galactosidase. Both the immobilized and the native enzyme showed an optimum activity at 42°C.

Kinetic constants: The immobilized β-galactosidase followed simple Michaelis-Menten kinetics with both the natural (lactose) and synthetic (nitrophenylgalactopyranoside) substrates. The K_m values for the native and immobilized enzyme were for nitrophenylgalactopyranoside, 0.94 and 0.88mM, and for lactose, 10 and 9.7 mM, respectively (figures 1 and 2).

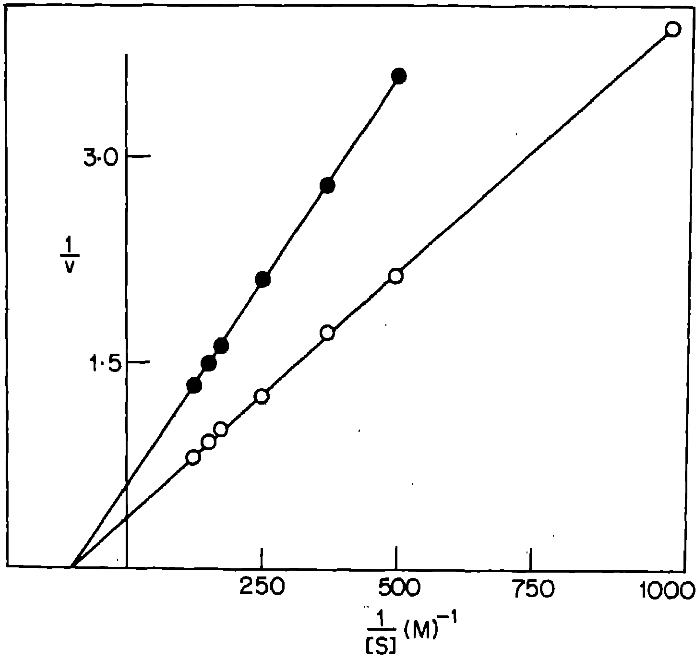

Figure 1 Lineweaver-Burk plots for lactose hydrolysis by native (O) and immobilized (●) β-galactosidase-

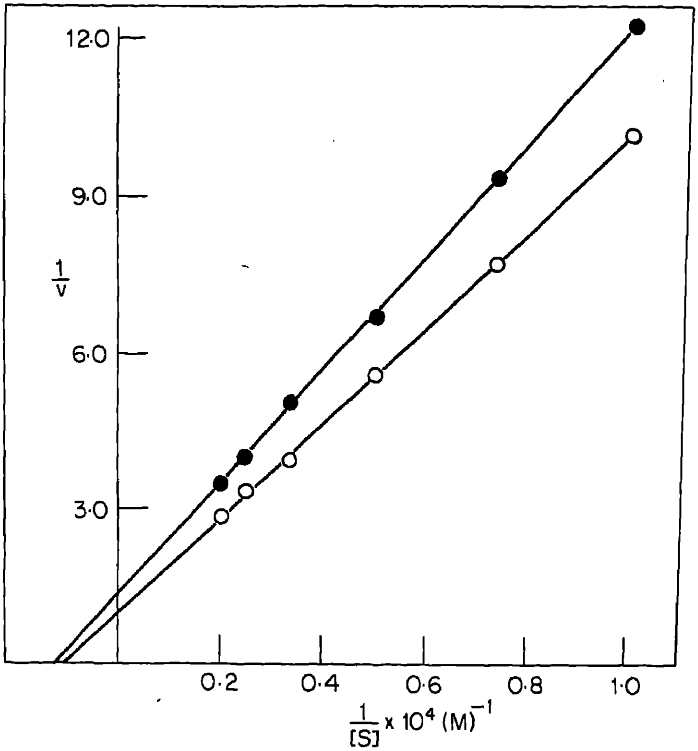

Figure 2 Lineweaver-Burke plots for nitrophenylgalactopyranoside hydrolysis by native (O) and immobilized (●) β-galactosidase

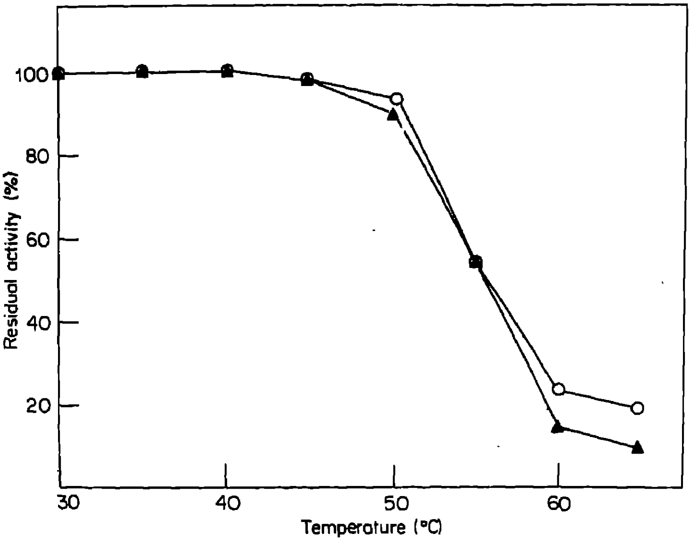

Figure 3 Heat stability of native (▲) and immobilized (O) β-galactosidase. The native or the immobilized β-galactosidase was kept at the mentioned temperature for 10 min, rapidly cooled, and the residual activity measured as mentioned in the text.

Heat stability: The heat stability of the immobilized enzyme was essentially the same as that of the native enzyme (figure 3). Both the forms of the enzyme started losing activity above 40°C. At higher temperature (60°C and 65°C) the immobilized enzyme was found to be slightly more stable than the native enzyme.

pH stability: The pH stability of immobilized β-galactosidase was almost the same on the alkaline side. However, the difference on the acidic side was more marked. It varied from 46% to 15% in the pH range of 4.5 to 6.0 (figure 4).

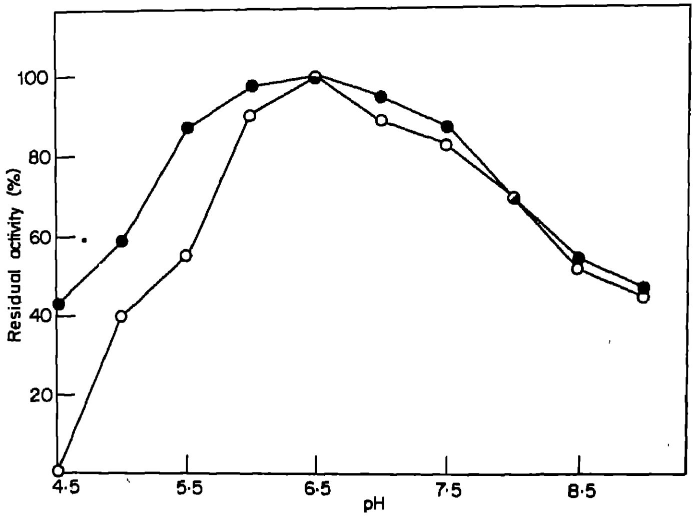

Figure 4 pH stability of native (O) and immobilized (●) β-galactosidase. The native or the immobilized β-galactosidase was brought to the mentioned pH value by the addition of acid or alkali with constant stirring on a magnetic stirrer to minimise the denaturation of enzyme due to 'localised' increase in acid or alkali concentration. These were kept at the mentioned pH for 10 min and then brought to pH 6.5. The residual activity was measured as mentioned in the text.

Stability of immobilized enzyme during storage: No loss in enzyme activity was observed for 30 days when the immobilized β-galactosidase preparation was stored at 4°C. A 30% loss in activity was observed after 60 days of storage. However, the native enzyme showed a 60% loss in activity after 60 days of storage. For practical applications the immobilized enzyme might have to be stored at the ambient temperature (~25°C), instead of 4°C. So the immobilized enzyme preparation was stored at 25°C and tested daily for activity. No loss in the activity of the preparation was found up to 20 days of storage. About 27% loss in activity was found for the native enzyme when stored for 20 days at 25°C.

Stability of immobilized enzyme after repeated use: The stability of the immobilized enzyme after repeated use was investigated in a batch assay. The immobilized enzyme preparation could be used repeatedly 12 times without any loss of activity.

Hydrolysis of lactose: Four grams (wet weight) of immobilized enzyme preparation was incubated at 42°C in 50 ml of 4.5% buffered lactose solution (pH 6.5) with continuous shaking. The glucose released was estimated upto 5 h of incubati At 2.5 and 5.0 h of incubation period 20% and 32% of lactose was hydrolyz respectively.

Discussion

The maximum yield of the immobilized enzyme was obtained when 2 g acrylami was used but higher concentrations resulted in lower yields. A similar trend w observed for the β-galactosidase of *E. coli* K 17 when entrapped in polyacrylami (Bunting and Laidler, 1972). It is quite likely that the decrease in activity yield the immobilized enzyme with increase in acrylamide concentration is due to inac vation of β-galactosidase caused by acrylamide during immobilization.

The yield of the immobilized enzyme depended on the amount of enzyme us for the immobilization as shown in table 2. Maximum activity yield was observ at 3 mg of enzyme used. It appeared that the amount of enzyme trapped within t gel decreased as the amount of enzyme added in the reaction mixture w increased beyond 3 mg. It is difficult to explain these results satisfactorily diffusional restrictions were overcome by shaking the reaction mixture.

Under the standard conditions of immobilization, the maximum activity yield immobilized enzyme was 31%. A similar observation was made by Ohmiya *et* (1975) who reported 28% activity yield of immobilized β-galactosidase fro *Aspergillus oryzae*. However, 48% yield of activation during the entrapment of galactosidase from *Saccharomyces lactis* was observed (Dahlqvist *et al.*, 1973).

The activity of immobilized enzyme was low when the reaction was catalyz without shaking. Increase in activity of the immobilized enzyme when t reaction mixture was stirred showed that the reaction was diffusion-controlle Bunting and Laidler (1972) also observed during their studies on β-galactosidase *E. coli* K17 that the reaction was diffusion-controlled when it is entrapped in po acrylamide gel, especially when the slices were thick. In our studies t diffusional effects were not observed when the reaction mixture was stirre Kobayashi and Moo-Young (1972) predicted that Lineweaver-Burk plots for t immobilized enzyme where the reaction is diffusion-controlled is convex to the axis and the same was observed by Bunting and Laidler (1972). No curvature w observed in Lineweaver-Burk plots for the immobilized enzyme in our studie which implies that the reaction was diffusion-free under our conditions when t reaction mixture was stirred at 100 rpm. On comparing the properties of t immobilized β-galactosidase with those of native β-galactosidase of *L. bulgaricus*, is evident that entrapment of the enzyme did not bring about any change in t optimum pH and the K_m. According to the hypothesis of Kobayashi and Laidl (1974) immobilization of an enzyme does not bring any change in optimum pH a K_m values when the charge on the matrix used for immobilization is nil and diff sional effects are negligible. Polyacrylamide gel does not have any charge and t small diffusional effect present in this case has been checked by stirring t reaction mixture as mentioned above, so it is reasonable to observe no change pH optimum and K_m values. However, an unexpected and as yet unexplain shift of the optimum pH of the immobilized aminoacylase by 0.5 pH units to t acid side in comparison with that of the native enzyme was observed by Mori *et* (1979). A similar shift was reported for the immobilized phosphoglycerate muta in an acrylamide gel lattice (Bernfield *et al.*, 1969). The pH stability of tl immobilized β-galactosidase was found to be higher than that of native β-galact sidase. The difference was significant in the acidic range (figure 4).

No marked difference was observed in the optimum temperature and therm stability between immobilized and native β-galactosidase. Available reports (Mo *et al.*, 1972; Ohmiya *et al.*, 1975) show that the optimum temperature and therm stability of the enzyme immobilized by entrapment in acrylamide gel are almo similar to those of the native enzyme. However, the immobilized β-galactosidas of *L. bulgaricus* was found to be slightly more thermostable than free galactosidase at 60°C-65°C, probabably because of stabilization of the conform tion of the enzyme molecules by the matrix at higher temperature.

The enzyme in the entrapped form is stable for 30 days at 4°C. Also no loss i the activity of the immobilized β-galactosidase was found upto 20 days when store at the ambient temperature (~25°C). These results indicate that immobilized galactosidase from *L. bulgaricus* can be used at ambient temperature over a goo period of time. Moreover, the stability after repeated use observed in our studie suggested that the β-galactosidase from *L. bulgaricus* in the entrapped form can b of great practical use.

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Effect of malathion exposure on some physical parameters of whole body and on tissue cations of teleost, *Tilapia mossambica (Peters)*

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Abstract. Exposure of fish to a sublethal (2 mg/litre/48 h) concentration of malathion showed no significant changes in any of the physical parameters investigated like, body weight and body water content, while the oxygen consumption of the fish showed a consistent increase upto 24 h and later declined to 8% suggesting the reduction of oxidative metabolism at the end of 48 h. The sodium, potassium and calcium ions showed a remarkable decrease in all the tissues of malathion-exposed fish, suggesting the loss of ions from the body of these fish. The results suggested that the regulation of osmotic balance may occur by an effective operation of the salt pump rather than the water pump.

Keywords. Oxygen consumption; ions; malathion; *Tilapia mossambica*

Introduction

Living organisms have the ability to adapt themselves to changes in the environment such as temperature, humidity, oxygen supply or a pesticide stress (Hoar, 1976). In some cases, especially in fish they may resist the change for a period of time, but will eventually succumb as a result of the inability to adapt to the change (Fry, 1971). The effect of an organophosphorus pesticide, malathion, was studied on some parameters associated with the physiology of fish exposed to tolerable concentrations of malathion (2 mg/L). In order to examine the sequence of changes associated with body weight, water content and oxygen consumption of malathion-exposed fish, investigations were also conducted at 12 h intervals after exposure to malathion. Further, to gain an insight into the ionic fluxes, the levels of sodium, potassium and calcium were estimated in selected tissues of fish exposed to a sublethal concentration of malathion for 48 h.

Materials and methods

Immature fish, *Tilapia mossambica* (8±2g, size 6±1 cm) were collected from the local ponds and acclimated to laboratory conditions. They were fed daily with groundnut cake and frog leg muscle twice a week. A stock solution of 1000 ppm (mg/ml) malathion was prepared in acetone. Required dilutions of the ac formulation were made with tap water. Acetone in the quantity used was not to fish (Pickering *et al.*, 1962). After determination of LC$_{50}$ (*i.e.* the concentr which produced 50% lethality) 5.8 ppm (Kabeer and Ramana Rao, 1980), f batches of 6 were exposed to 48 litres of 2 ppm (sublethal) malathion for (Kabeer, 1979). For the determination of changes in body weight of the fish, fish was taken out of the tap water, wiped dry with filter paper, numbered a fresh weight recorded quickly in an electrical balance. The fish exposed to 2 malathion were taken out at 12 h intervals, weighed and put back in the medium. To estimate the water content of the body, six each of norma malathion exposed (2 mg/litre/48 h) fish were wiped dry and weighed. They dried in a hot-air oven at 80°C for 24 to 48 h. After drying, the weight of th was recorded and the drying was continued until no further change in weigh observed. The change in water content was expressed as g/g fish. The o consumption of the normal and malathion exposed fish was estimated b Winkler's iodometric method as described by Welsh and Smith (1953) and S (1959). The oxygen consumption was estimated in unit metabolism, choosi of oxygen consumed/g fish/h. For estimation of ions, the muscle, gill and tissues of normal and malathion exposed fish were wet-ashed (Dall, 1967 sodium, potassium and calcium were estimated using an Elico flame photon The values were expressed in mmol/g wet wt of tissue. Statistical evaluation data was done by the Student 't' test (Snedecor, 1956).

Results and discussion

The body weight of malathion exposed fish showed a slight but progr decrease in the time course when compared with normal fish (table 1), suggest the loss of some body constitutents. Since the loss of weight was pro associated with the susceptibility to pesticides (Gish and Chure, 1970), the longed exposure of fish to the same concentration of malathion may prove fatal. Studies with other organophosphorus compound like methyl parathi the fish *Tilapia mossambica* (Siva Prasada, 1980) and malathion, sevin and lir on the same species (Basha, 1980) showed a decrease in the body weight.

There was no change in the water content of the normal and malathion ex fish (table 1). It is likely that this dynamic state of the treated fish might be d the prevalance and maintenance of osmotic balance during malathion expo This osmotic balance might be the result of the salt pump rather than the pump from the body to the medium, aimed towards maintaining the structura functional integrity of the cell (Hoar, 1976) following malathion exposure.

Nevertheless, the rate of oxygen consumption increased significantly duri first 24 h of exposure, but decreased thereafter though this was statisticall significant (table 1). The initial increase in the rate of oxygen consum suggests the acceleration of oxidative metabolism which after 24 h declines compared to normal fish. This decline in oxygen consumption is in conso with the diminished levels of oxidative enzymes after 48 h of malathion expos observed earlier (Kabeer, 1979). Since symptoms of pesticide toxicity nor involve respiratory distress (Ferguson and Goodyear, 1967), the decreased oxyge consumption of the malathion exposed fish is probably due to the absorbance more pesticide through the gills (Ferguson *et al.*, 1966).

Table 1. Effect of malathion exposure on fresh weight, water content and oxyg consumption of *Tilapia mossambica.*

	Exposure (h)							
	12		24		36		48	
	Normal	ME^a	Normal	ME^a	Normal	ME^a	Normal	ME^a
Fresh weight (g)								
	10.22 ±0.355	10.10 ±0.395 −1.17%	9.87 ±0.419	9.72 ±0.334 −1.519%	9.74 ±0.843	9.40 ±0.378 −3.49%	9.94 ±0.862	9.36 ±0.37 −5.84
Water content ^{b}								
	0.781 ±0.0502	0.779 ±0.0493 −0.256%	0.750 ±0.0394	0.755 ±0.0393 −0.667%	0.786 ±0.0495	0.775 ±0.0641 −1.399%	0.771 ±0.748	0.76 ±0.07 −6.25
Oxygen consumption ^{c}								
	0.2516 ±0.0084	0.3139* ±0.0075 +24.76%	0.2442 ±0.0087	0.2717* ±0.0123 +11.26%	0.2795 ±0.0099	0.2644 ±0.0266 −5.40%	0.1814 ±0.0214	0.16 ±0.01 −7.72

Each value is the mean ±S.D. of 6 individual observations. + or − indicates the per cen increase or decrease over normal, respectively. * Signifies values statistically significant.

^{a} Malathion exposed; ^{b} g/gfish; ^{c} ml O_2 consumed/g fish/h.

The sodium (Na$^+$), potassium (K$^+$) and calcium (Ca^{2+}) ions in the muscle, gi and liver tissues of 48 h malathion exposed fish decreased as compared to norma tissues (table 2). Except the Ca^{2+} content in the muscle and liver tissues, th decrease in Na$^+$, K$^+$ and Ca^{2+} ions of the three tissues was found to b statistically significant (table 2). A similar decrease in the Na$^+$, K$^+$ and Ca^2 content was reported in the liver tissues of fish exposed to endrin (1 mg/litre/96 (Eisler and Edmunds, 1966). The decreased ionic content of the malathio exposed tissues evidently suggest a change in the permeability properties different biological membrane systems. It was observed that osmotically activ ions like amino acids (Kabeer, 1979) and metabolites like pyruvate, lactate an acetate may be on the increase under stress (Hoar, 1976). This results in th hypertonicity of the animal tissues during malathion stress. However it is obvio that the malathion exposed fish seem to prefer the salt pump (pumping out Na K$^+$ and Ca^{2+}) rather than the water pump since negligible change in the wat content of the whole animal was observed (table 1). In general, it is obvious th the malathion exposed fish maintain the isoosmotic condition of the intracellul milieu by pumping out the Na^+, K^+ and Ca^{2+} ions and try to compensate this by enhancing the amino acid pool (Kabeer, 1979), without affecting the wa content of the body during malathion stress.

Table 2. Effect of malathion exposure on sodium, potassium and calcium contents in t muscle, gill and liver tissues of *Tilapia mossambica*.

	Muscle		Gill		Liver	
	Normal	ME [a]	Normal	ME [a]	Normal	ME
Sodium [b]						
	0.0529 ±0.0026	0.0468** ±0.0034 −11.53%	0.1096 ±0.0096	0.0883* ±0.0041 −19.43%	0.0700 ±0.0087	0.05 ±0.00 −19.0
Potassium [b]						
	0.0599 ±0.0031	0.0492* ±0.0037 −17.86%	0.0700 ±0.0030	0.0598* ±0.0021 −14.57%	0.0582 ±0.0033	0.04 ±0.00 −41.2
Calcium [b]						
	0.0040 ±0.0008	0.0033 ±0.0015 −17.5%	0.0097 ±0.0011	0.0077** ±0.0016 −20.62%	0.0034 ±0.0005	0.00 ±0.00 −23.53

Each value is the mean ±S.D. of 6 individual observations. —indicates the per cent decre over normal. * =Significant at $P < 0.001$. ** =Significant at $P < 0.005$.

[a] Malathion exposed; [b] mmol/g wet wt tissue.

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The inhibition of photosynthetic electron transport by methyl parathion

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Abstract. The effect of methyl parathion (metacid-50), an organophosphorous insecticide, on the Hill reactions of isolated mesophyll chloroplasts of *Sorghum vulgare* was studied. The pesticide was found to inhibit the Hill reaction with all the Hill oxidants tested, namely potassium ferricyanide, 2,6-dichlorophenol indophenol and *para*-benzoquinone. The concentration of the pesticide required to inhibit 50% of the control Hill activity (I_{50} value) was found to vary with the different Hill oxidants.

Keywords. Chloroplast electron transport; methyl parathion; Hill reaction.

Introduction

Insecticides are being increasingly used to improve agricultural production. These insecticides, besides effectively controlling pest attack, may affect plant photosynthesis and thereby decrease photosynthetic productivity to a considerable extent. In fact, the once widely used organochlorine insecticides have been shown to exhibit inhibitory effects on photosynthetic processes (Bowes and Gee, 1971; Bowes, 1972). Organochlorine insecticides are now being replaced mostly by organophosphorous insecticides (Fest, 1977) and so far no study has been undertaken to find out whether this new group of pesticides has any effect on the photosynthetic process. The present report shows that methyl parathion, one of the most widely used organophosphorous insecticides inhibits photosynthetic electron transport in isolated chloroplasts.

Materials and methods

All the chemicals used were of analar grade. They were obtained from BDH, Bombay (sorbitol and NaCl), Sarabhai. M Chemicals, Baroda ($K_3[Fe(CN)_6]$, $MgCl_2$, $MnCl_2$, sodium phosphate mono- and dibasic) and from BDH, Poole, England (DCPIP and DPC). Benzoquinone was prepared in the laboratory by the oxidation of hydroquinone (BDH, Bombay) with potassium bromide.

Abbreviations used: DCPIP, Dichlorophenol indophenol; DPC, Diphenyl Carbazide.

Isolation of chloroplasts

Sorghum vulgare CV20 was grown in the University botanical garden und natural conditions. The chloroplasts were isolated by grinding 10-day o *Sorghum* leaves in 50 mM phosphate buffer, pH 6.5, containing 400 mM sorbitol. mM EDTA; 10 mM NaCl, 5 mM $MgCl_2$ and 1 mM $MnCl_2$ in a Sorvall Omnimixer 50% line voltage for 5 s thrice with 5 s gaps in between. The homogenate w filtered through two layers of nylon cloth of 100 µ mesh size. The filtrate w centrifuged at 2000 *g* for 2 min, the chloroplasts pellet washed once with t grinding buffer and resuspended in the same buffer.

Assay for Hill reaction

The reaction mixture for the Hill reaction contained 50 mM phosphate buffer 7.5, 100 mM sorbitol, 2 mM EDTA, 5 mM $MgCl_2$, chloroplasts equivalent to 20- µg of chlorophyll per ml of reaction mixture and any one of the Hill oxidants nam potassium ferricyanide 1 mM, dichlorophenolindophenol 50 µM, or benzoquino 2.5 µM. The light-dependent oxygen evolution with a Hill oxidant was taken a measure of Hill activity (Trebst, 1972).

The oxygen evolution was followed polarographically under saturating li (>620 nm) using a YSI 4004 Clark oxygen electrode (Yellow Spring Instrume Co., Yellow Spring, Ohio, USA) hooked to a Heath (Model EU 20B) ser recorder. The chloroplasts prepared in the above manner were found to be coupled as evidenced by the absence of any effect by NH_4Cl.

Assay for PS II reaction with artificial electron donor

The PS II reaction was assayed with an artificial electron donor (0.5 mM diphen carbazide) after inactivating the water splitting system by heating the chloropla for 2 min at 50°C. Dichlorophenolindophenol was used as an electron accept (Yamashita and Butler, 1968).

Light-dependent reduction of dichlorophenolindophenol was assayed phot metrically by monitoring the decrease in the absorbance at 610 nm using Spectronic 70 (Bausch and Lomb) spectrophotometer (Trebst, 1972).

The reaction mixture was exposed to actinic light for 30 s and the actual amou of dichlorophenolindophenol reduced was estimated using an extinction coef cient value of 20 mM^{-1} (Trebst, 1972).

Estimation of chlorophyll

Chlorophyll was extracted in 80% acetone and estimated according to Arn (1949).

Insecticide used

The commercial product Metacid-50 (R) (Bayer India Limited, Thana, Bomba containing 50% active ingredient of methyl parathion (50% dimethyl *p*-nitrophen thiophosphate) and also the active ingredient of methyl parathion were used in these experiments.

Results and discussion

Figure 1 shows the inhibition of Hill activity of isolated, broken *Sorghum vulgare* mesophyll chloroplasts by methyl parathion with the three different Hill oxidants.

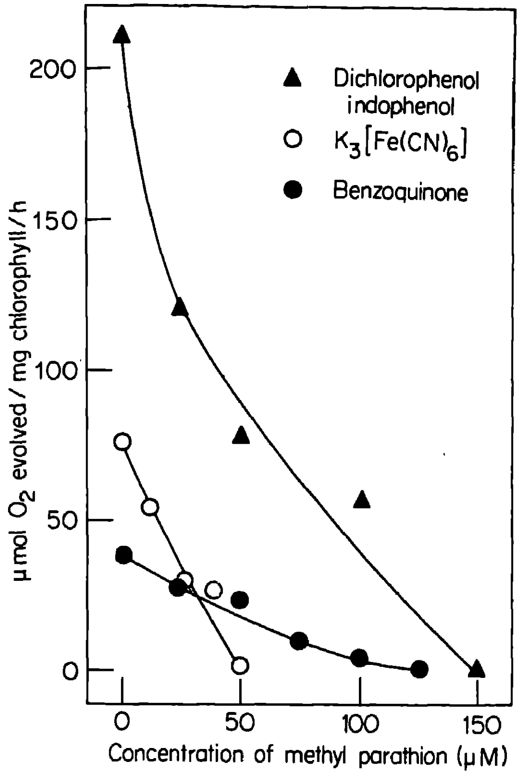

Figure 1. Effect of methyl parathion on the Hill reaction in isolated chloroplasts, with different Hill oxidants.

Methyl parathion was found to inhibit the Hill activity with all the Hill oxidants tested, although the concentration of the methyl parathion required for 50% inhibition (I_{50} value) varied with the different Hill oxidants (figure 1).

This inhibitory action of the organophosphorous insecticides (I_{50}: 25-47 µM) was considerably weaker than that of 3-(3,5-dichlorophenyl)-1, 1-dimethylurea (I_{50}: 0.05 µM) or atrazine (I_{50}: 0.5 µM) which are potent inhibitors of the Hill reaction (Izawa and Good, 1972). However methyl parathion is comparable with other Hill reaction inhibitors such as *O*-phenathroline which has an I_{50} at 10 µM (Izawa and Good, 1972).

The Hill reaction in chloroplasts can be inhibited in two general ways, either by blocking the electron transport between the two photosystems or by impairing the water splitting system. The observation that the I_{50} value for the inhibition of the Hill reaction by methyl parathion varies with the different oxidants indicates that methyl parathion inhibits the electron transport between PS II and PS I without affecting the water splitting system. This possibility was verified with diphe carbazide as an artificial electron donor to the PS II reaction centre a inactivating the water splitting system by heat treatment (table 1). The effec

Table 1. Inhibition of the PS II reaction by methyl parathion.

Components of reaction mixture	μmol DCPIP[a] reduced/mg chlorophyll/h
Chloroplast + DCPIP[a]	51.92
Chloroplast + DCPIP + Methyl parathion (10/μM)	3.54
Heat-treated chloroplast + DCPIP	0
Heat-treated chloroplast + DCPIP + DCP[b]	16.52
Heat-treated chloroplast + DCPIP + DPC + methyl parathion (100 μM)	0

[a] Dichlorophenol indophenol; [b] Diphenyl carbazide.

The PS II reaction was assayed with diphenyl carbazide (DPC) as an electron donor to the PS I reaction centre after inactivating the water splitting system by heat treatment.

the commercial methyl parathion on photosynthetic electron transport in isol chloroplasts is entirely due to methyl parathion and not because of some o substances present in it. This has been confirmed using the active ingredie methyl parathion where the same effect as that of the commercial product noted (results not shown).

The inhibition of photosynthetic electron transport in isolated chloroplas methyl parathion leads to the logical question of whether methyl parathion a level present in the environment has any effect on the photosynthetic prc under field conditions.

For methyl parathion to have any direct effect on photosynthesis under conditions, it should first enter chloroplasts and persist there for quite some t There are reports that the methyl parathion enters the plant and is transloc inside it (Coeffin, 1964; Attri and Rattan Lal, 1974c; Kannan and Jayaraman, 1 But these organophosphorous insecticides are known to be degraded very ra within the plants (Agnihotrudu and Mithyanantha, 1978). Thus when it is spr at the concentrations of 250-400 ppm the residue level in the plant drops down 5 ppm on the second day and reaches a non-detectable level in about ten days (Attri and Rattan Lal, 1974a, b). As shown in figure 1 the concentration of m parathion required to inhibit 50% of the Hill reactions of isolated chloroplast the range of 25 to 47 μM (16-29 ppm). Thus, under field conditions, as a res the quick degradation of methyl parathion, such a concentration may be r only for one or two days after spraying. However, a short term effect immedi after spraying may still be considerable.

This circumstantial evidence suggests that under the field conditions, methyl parathion may not inhibit the photosynthetic process as long as it is sprayed at the optimal concentration. However when this pesticide is sprayed in higher concentrations and/or non-uniformly, the photosynthetic process may be inhibited to a considerable extent. These results highlight the danger of indiscriminate spraying and pinpoint the need for caution in using this insecticide.

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Structure of presynaptic toxins: Crystallization and preliminary x-ray diffraction data on Notechis II-5, a presynaptic toxin phospholipase

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Abstract. The presynaptic neutrotoxin-phospholipase, Notechis II-5 from the venom of Notechis *Scutatus scutatus* (Australian tiger snake) has been crystallized in a form suited for x-ray diffraction analysis. The crystals belong to the orthorhombic space group $P2_1 2_1 2_1$ with unit cell dimensions, $a=146.1$, $b=43.5$ and $c=39.0$ Å. There are two molecules of Notechis II-5 in the asymmetric unit. The molecular weight is about 13,500. Notechis II-5 is highly homologous to Notexin, another presynaptic toxin from the venom of the Australian tiger snake, to bovine and porcine pancreatic phospholipases A and other venom phospholipases.

Keywords. Notechis II-5; Australian tiger snake; presynaptic toxin; crystallization; x-ray diffraction.

Introduction

The characteristic paralysis caused after snake bite is due to the blocking of the nerve transmission across the cholinergic neuromuscular junction by protein toxins present in the snake venom (Eaker *et al.*, 1976). Two categories of neurotoxins have been characterized on the basis of their site of activity. Post-synaptic toxins show curare-like action and the nicotinic acetylcholine receptors of the muscle motor end plate. These proteins consist of about 70 amino acids and the three-dimensional structures of some of this group of neurotoxins have been well established and their function has been well characterized (Low *et al.*, 1976; Tsernoglou and Petsko, 1976). Presynaptic toxins interfere with the release of acetylcholine from the motor nerve terminals. The presynaptic toxins fall into two classes (a) multimeric protein complex and (b) monomeric protein. All these toxins exhibit pronounced phospholipase activity. In the case of the multimeric toxins, the phospholipase activity is usually associated with one of the sub-units and is an essential prerequisite for the toxicity. The nature of the presynaptic toxicity and the need for the phospholipase activity are not well understood.

We have investigated the structure-function of the monomeric presynaptic toxins by x-ray diffraction work. Earlier we had successfully reported the crystallization and x-ray data on Notexin isolated from the venom of the Australian tiger snake, Notechis *Scutatus scutatus* (Kannan *et al.*, 1977). In this paper we r the crystalization and the crystallographic data on another presynaptic Notechis II-5 isolated from the same Australian tiger snake venom (Halper Eaker, 1976).

Notechis II-5 is highly homologous to Notexin and to porcine, bovine and ve phospholipases. It shows moderate to high phospholipase activity and is 20 less neurotoxic compared to Notexin. These toxins consist of about 119 a acids and have a molecular weight of about 13,500. The homologous pho lipases do not exhibit any toxicity. It would thus be useful to correlate the te structure of these proteins with their toxicity and phospholipase activi compared to the non-toxic phospholipases from different species.

Crystallization

The purification of Notechis II-5 has been described by Eaker and co-wo (Halpert and Eaker, 1976). The purified and lyophilized powder was suspenc 0.05 M Tris sulphate buffer (pH 8.5) and a few drops of 1 M ammonium ac were added to the suspension until the precipitate just dissolved. The solutio left at 4°C and crystals were obtained within a short period. The crysta rectangular plates having dimensions $0.3 \times 0.3 \times 0.5$ mm^3, diffract well an stable to x-rays.

Results and discussion

The crystals were mounted in the usual way in Lindemann capillaries an precession photographs (figures 1 and 2) were collected on a 600 watt Philip focus tube using Ni filtered CuK$_\alpha$ radiation. The photographs exhibited syste absences along the h00, 0k0 and 00l directions. The unit cell parameters de

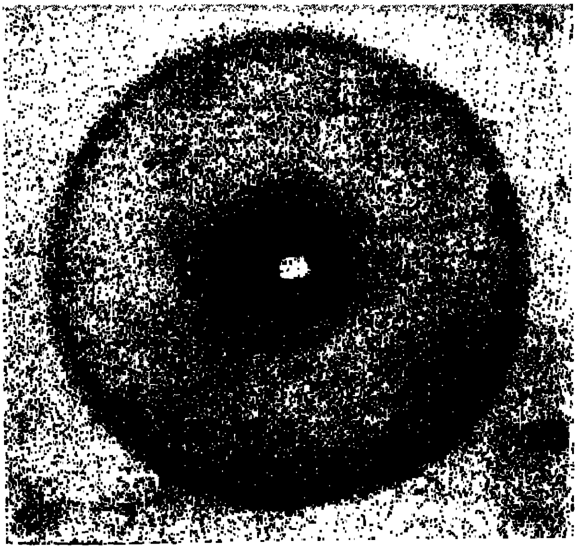

Figure 1. HKO photograph of Notechis II-5. Precession angle 12°.

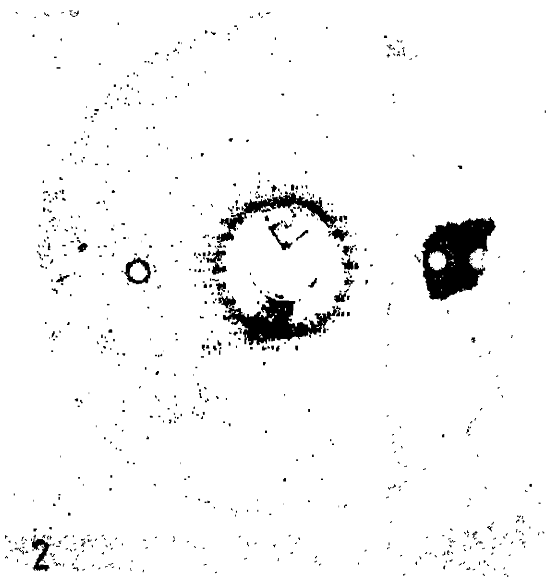

Figure 2. HK1 photograph of Notechis II-5. Precession angle 12°.

from the diffraction photographs are $a=146.1$, $b=43.5$ and $c=39.0$ Å^{3}and the space group is $P2_12_12_1$. The volume is 247,643 Å^3 giving a Mathews number (Mathews, 1968) $V_M=4.5$ Å^3/Dalton. This value is very much higher than the normally observed one of 2.3 Å^3/Dalton for one Notechis II-5 molecule per asymmetric unit. The homologous Notexin crystals gave a Mathews number of $V_M=3.05$. It is thus evident that the unit cell of Notechis II-5 crystals consists of a dimer of molecular weight 27,000 in the asymmetric unit, which would give a Mathews number of 2.25 Å^3/Dalton, a more acceptable value for globular proteins.

The homologous phospholipase A2 crystallized from *Crotalus adamanteus* and *C. atrox* have a Mathews number of 2.28 and 2.21 respectively, for a dimer of molecular weight 30,000 in the asymmetric unit (Pasek *et al.*, 1975). This is analogous to Notechis II-5 reported here. Further work is in progress to get heavy atom derivatives.

Acknowledgements

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Action of 2,4-dichlorophenoxyacetic acid and rifampicin onheterocyst differentiation in the blue-green alga, *Nostoc linckia*

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Abstract. 2,4-Dichlorophenoxyacetic acid, a commonly used herbicide, increased the growth of the filamentous blue-green alga, *Nostoc linckia* at doses upto 100 µg/ml. The herbicide-treated N_2-cultures showed enhanced heterocyst frequency and N_2-growth. Thus, the herbicide stimulated algal growth at the expense of molecular nitrogen under aerobic growth conditions. Rifampicin caused chain formation of heterocysts. This was effectively counteracted by 2,4-dichlorophenoxyacetic acid, suggesting a biological interaction between them at the level of the heterocyst spacing control mechanism.

Keywords. Blue-green alga; heterocyst differentiation; N_2-growth, rifampicin; 2,4-dichlorophenoxyacetic acid.

Introduction

The hormone weed killer 2,4-dichlorophenoxyacetic acid is generally used to control the excessive growth of aquatic weeds in lakes and inundated areas. Blue-green algae, particularly nitrogen-fixing genera which constitute the principal components of microbial flora in waterlogging paddy field soils, play an important role in the productivity of such ecosystems through carbon and nitrogen fixation (Singh, 1961; Stewart *et al.*, 1978). It was earlier reported that these algae show a relatively high tolerance towards pesticides (Venkataraman and Rajyalakshmi, 1972; Singh, 1974; DaSilva *et al.*, 1975) but higher concentrations of 2,4-dichloropehnoxyacetic acid (above 1000 µg/ml) are reported to be inhibitory to nitrogen fixation by these organisms (Lundkvist, 1970; Das and Singh, 1977).

The exact mode of 2,4-dichlorophenoxyacetic acid action in biological systems is not known. However, it was reported to inhibit oxygen uptake by mitochondria and oxygen evolution by chloroplasts besides interfering with nucleic acid metabolism and protein synthesis (Ashton and Crafts, 1973). Such studies were not carried out with blue-green algae, although they served as excellent tools for the examination of the biological effects of the herbicide on heterocyst differentiation and functions. Rifampicin, a well-known inhibitor of DNA-dependent RNA polymerase in bacteria, has been reported to induce the formation of chains of heterocysts in the nitrogen-fixing filamentous *Anabaena variabilis* (Wolk Quine, 1975) presumably by interfering with the control mechanism of hetero spacing pattern. Our initial aim was to investigate the biological implication o use of 2,4-dichlorophenoxyacetic acid in rice field ecosystems dominate nitrogen fixing blue-green algae, by examining its effect on N_2 growth and het cyst differentiation in *Nostoc linckia* under cultural conditions. We have trie understand the possible mode of 2,4-dichlorophenoxyacetic acid action by usi in combination with rifampicin, an inhibitor of transcription in procaryotes.

Materials and methods

Organisms and growth conditions

The experimental alga, *Nostoc linckia*, is a heterocystous and nitrogen fi paddy soil blue-green alga which was employed in the present study (Singh Tiwari, 1969). The clonal and axenic population of the alga was maintaine cultures by serial transfers in modified Chu-10 nitrogen-free medium (Saffer and Morris, 1964) with addition of A_6 as microelements (Allen and Arnon, 19 Chu-10 is designated as a culture medium after its discoverer, Chu (1942). cultures were grown in a room lit with daylight fluorescent tubes emitting 2,200 light intensity for 14 h a day at 24 ± 1°C. The nitrogen-free medium (C-N) sup mented with $Ca(NO_3)_2$ and NH_4Cl(0.01M) is designated as C+NO_3 and C+ respectively. The stock solution of sodium salt of 2,4-dichlorophenoxyacetic (obtained from Pesticide India, Udaipur, with 80% active ingredient) and rifamp (Sigma Chemicals Company, St. Louis, Missouri, USA) were prepared in ste nitrogen-free medium and sterile water, respectively and added to the des concentration in a final volume of 20 ml medium after filter sterilizat Similarly, 3(3,4-dichlorophenyl)-1, 1-dimethyl urea (dichlorophenyl dimethyl u was dissolved initially in ethanol before diluting 100 times in the medium in orde get final concentration of 2.0 μg/ml.

Growth measurements and heterocyst differentiation

Exponentially growing algal cultures were harvested by centrifugation (5000 *g*) washed twice with sterile distilled water. After resuspending in sterile medi aliquots of 0.5 ml were inoculated in 20 ml of the test medium in 100 ml Erlenm flasks with attached sidearms. Growth experiments were also conducted v dichlorphenyl dimethyl urea, a photosynthetic inhibitor of pigment system I the presence of 2,4-dichlorophenoxyacetic acid in order to determine whet these chemicals could be photoheterotrophically utilized as organic carbon for growth of the alga. Since our initial aim was to study heterocyst differentiatio the above growth conditions along with rifampicin supplements, the ammoni grown, undifferentiated filaments were used as inocula in all experiments. growth was measured in terms of the turbidity of cultures with a Photochem col meter using a red filter (650 nm) at regular intervals. Acetone and water sol pigments were extracted by the methods described by Allen and Smith (1969) the absorbance was recorded at 660, 460 and 620 nm, respectively for chlorophy carotenoids and phycocyanin contents. The growth rate constant (k) was calcu ed from the equation $k_t = \log_{10}(N_t/N_0)$; where k=growth rate constant, t=10 d

N_t=absorbance at time t and N_0=absorbance at time 'O' (Kratz and Myers, 1955). The 2,4-dichlorophenoxyacetic acid sensitivity of the alga was also tested on 1% Bacto-Difco agar nutrient medium containing graded concentrations of the herbicide. Heterocyst frequency was determined by counting the number of heterocysts per 100 vegetative cells under the microscope.

Estimation of total nitrogen

For the estimation of total nitrogen fixed by the alga under the above set of conditions, the alga was grown in 50 ml C-N medium (*i.e.* Chu-10 nitrogen-free medium) containing different concentration of the herbicide. Total nitrogen was finally (after 15 days) estimated by the microKjeldahl method (Piper, 1966) and the values were compared with those of control cultures.

Results

The specific growth rate (k) was determined in C-N, C+NO_3 and C+NH_4 medium in the presence of varying concentrations of 2,4-dichlorophenoxyacetic acid (100-1500 µg/ml) at different incubation periods. The respective media without 2,4-dichlorophenoxyacetic acid served as controls. The k value for 100 µg/ml 2,4-dichlorophenoxyacetic acid was found to be greater than in the control cultures but it eventually decreased with increasing concentrations (figure 1). The alga could tolerate upto 1500 µg/ml of 2,4-dichlorophenoxyacetic acid in C-N medium and 2000 µg/ml served as a complete lethal dose as evidenced by the loss of turbidity and absence of filaments under microscopic observation. The growth

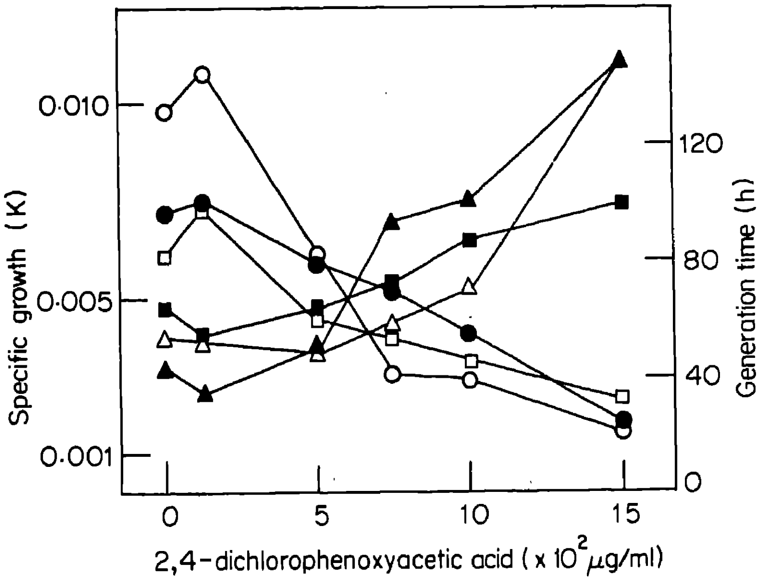

Figure 1. Effect of different concentrations of 2,4-dichlorophenoxyacetic acid on specific growth rate (k) of the alga in C-N (O), C+NO_3 (●) and C+NH_4 (□) media and generation time (Δ, ▲, ■) respectively.

patterns of the alga in $C+NO_3$ and $C+NH_4$ media were almost similar to that in C medium although the k values for the former media were slightly higher than tl for the latter medium. The generation time of the alga was also calculated in t above growth media (figure 1). Apparently, 2,4-dichlorophenoxyacetic ac seems to act as a stimulator of the algal growth. Hence on the basis of the report results with 2,4-dichlorophenoxyacetic acid, the herbicide cannot be regarded an inhibitor of algal growth in *N. linckia.*

The total fixed nitrogen and heterocyst frequency of the algal cultures in nitr gen fixing growth medium was very much reduced at the higher concentrations 2,4-dichlorophenoxyacetic acid but the growth stimulatory concentration of tl herbicide resulted in more total nitrogen and enhanced frequency of heterocys (figure 2). The heterocysts were detached from the parental filaments grown

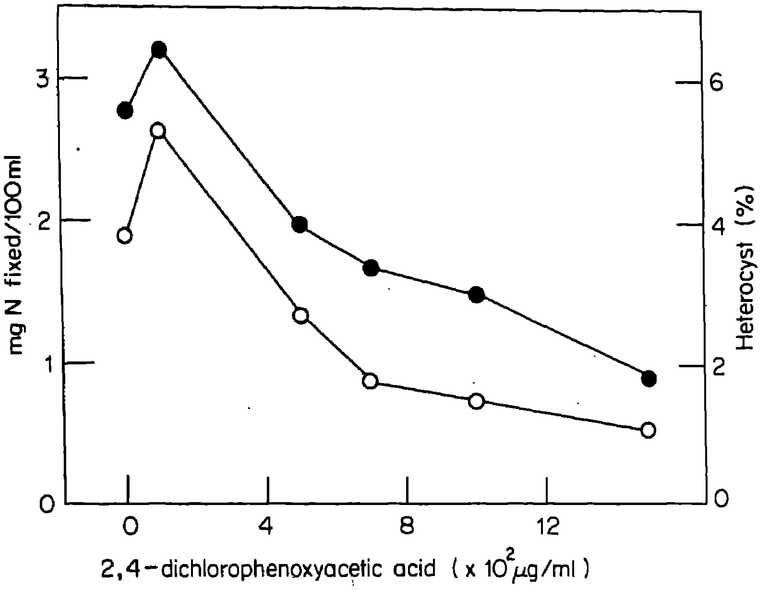

Figure 2. Effect of different concentrations of 2,4-dichlorophenoxyacetic acid on heterocyst frequency (●) and nitrogen fixation (O) in *Nostoc linckia.*

the elevated concentrations of the herbicide and the filaments grew, *albeit* poorl without heterocysts with much reduced level of cellular nitrogen. Dominance c blue pigmentation was pronounced in the above cultures in comparison to contr (table 1). However, exogenous supplementation of dichlorophenyl dimethyl ure in 2,4-dichlorophenoxyacetic acid medium completely inhibited the growth an dominance of blue-pigmentation of the alga. The filaments were fragmented int short pieces with no sign of heterocyst differentiation (table 2).

The effect of mixed supplementation of rifampicin and 2,4-dichlorophenoxy acetic acid was also studied on the pattern of growth and heterocyst differentiatio (table 1). Ammonium grown cultures were washed twice and inoculated into C-I

Table 1. Effects of 2,4-dichlorophenoxyacetic acid and rifampicin on heterocyst frequency and phycocyanin/chlorophyll *a* ratio in *Nostoc linckia*.

Concentration (μg/ml)	Heterocyst frequency (%)	Pigment ratio $\left(\frac{A_{620}}{A_{660}}\right)$
C−N[a] (control)	5.4	0.49
C−N+100 2,4-D[b]	6.5	0.55
C−N+100 2,4-D+0.1 R[c]	23.7	0.41
C−N+0.1 R	28.4	0.39
C−N+1000 2,4-D	2.1	0.45
C−N+1000 2,4-D+0.1 R	1.5	0.38

[a] C−N = Chu-10 nitrogen-free medium

[b] 2,4-D = 2,4-dichlorophenoxyacetic acid;

[c] R = rifampicin

medium containing either 2,4-dichlorophenoxyacetic acid or rifampicin or both in the medium. The concentration of 100 μg/ml of 2,4-dichlorophenoxyacetic acid enhanced the growth and heterocyst frequency of the alga but 1000 μg/ml partially inhibited both of them. Maximum heterocyst frequency was obtained at 100 μg/ml 2,4-dichlorophenoxyacetic acid with 0.1 μg/ml rifampicin complex medium and rifampicin medium alone whereas 1000 μg/ml 2,4-dichlorophnoxyacetic acid suppressed the effect of rifampicin on chains of heterocyst formation. The growth was, however, inhibited when 2,4-dichlorophenoxyacetic acid and rifampicin were combined at 1000 μg/ml and 0.1 μg/ml respectively. Thus, it was observed that rifampicin stimulated the heterocyst differentiation but 2,4-dichlorophenoxyacetic acid at higher concentrations inhibited heterocyst formation even in the presence of rifampicin (table 1).

Table 2. Effect of dichlorophenylmethyl urea and 2,4-dichlorophenoxyacetic acid on growth and heterocyst frequency in *Nostoc linckia*.

Medium (μg/ml)	Chlorophyll (A_{660})	Heterocyst frequency (%)
C−N (control)	0.05	5.4
C+100 2,4-D[a]	0.06	6.5
C+500 2,4-D	0.03	3.5
C+100 2,4-D+DCMU[b]	—	—
C+500 2,4-D+DCMU	—	—

[a] 2,4-D = 2,4-dichlorophenoxyacetic acid

[b] DCMU = dichlorophenol dimethyl urea

Discussion

Heterocyst differentiation and N_2 growth, the two biological parameters comm associated with the filamentous nitrogen fixing blue-green algae (Fogg *et al.*, 1 have been taken into consideration in the present study. As it is known nitrogen fixation in such algae is dependent on the development of specia cells, called heterocysts, under aerobic growth conditions, the increase in the nitrogen of the cultures grown at 100 µg/ml of 2,4-dichlorophenoxyacetic acid be correlated with the increase in frequency of heterocysts. Similarly, a ma decrease in N_2 growth of the alga at higher concentrations of the herbicide ca explained with much reduced frequency of heterocysts in the algal filaments. I heterocyst formation requires nitrogen starvation, organic carbon supply for wall synthesis and fresh protein synthesis (Haselkorn, 1978), a substance w interferes with meeting any one of the above requirements, would decrease formation of heterocysts.

2,4-Dichlorophenoxyacetic acid stimulates both heterocyst frequency an growth at a dose upto 100 µg/ml and is found to inhibit both the processes at hi doses. Therefore, it cannot be regarded as an inhibitor of N_2 fixation and het cyst formation. In fact, the results seem to suggest its activity as a stimulat heterocyst formation and N_2 growth. 2,4-dichlorophenoxyacetic acid is a synth growth hormone analogue and its effect on heterocyst frequency and N_2 gro may result from its metabolic utilization as a carbon source or from its inhibi the formation of heterocyst inhibitory substances involved in heterocyst spa control. Rifampicin, an inhibitor of transcription and consequently of growt found to cause chain formation of heterocysts, an observation similar to previously reported by Wolk and Quine (1975) for it and Mitchison and Wil (1973) for 7-azatryptophan in other heterocystous blue-green algae. The obse tions that rifampicin increases heterocyst frequency leading to the formatio heterocyst chains and 2,4-dichlorophenoxyacetic acid counteracts this aspect rifampicin-sensitive function in *Nostoc linckia* do indicate a kind of antagoni interrelation between these two inhibitors.

Acknowledgements

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Canavanine-induced inhibition of growth and heterocyst differentiation in *Anabaena doliolum* and isolation of a canavanine-resistant mutant

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Abstract. The effect of the arginine analogue, canavanine on growth and heterocyst differentiation in the nitrogen-fixing alga *Anabaena doliolum* has been studied. The analogue inhibited growth and heterocyst differentiation at a concentration as low as 1 μM. The treated algal cells lacked conspicuous granular inclusions, whereas treatment with chloramphenicol led to increased synthesis of granules (probably cyanophycin granules). Exogenously added arginine completely reversed the effect of the analogue but lysine could only partially relieve the effect. A time course study with canavanine indicated inhibition of fresh protein(s) synthesis at all steps where a new class of proteins is synthesized so that the action of the analogue does not seem to be specific for a particular kind of protein. A mutant resistant to this analogue has been successfully isolated indicating that this alga does not show mutational immunity at least to the amino acid analogues unlike in the observation with different antibiotics. Our observations indicate that canavanine either directly inhibits protein synthesis or forms defective protein(s) which produces all the observed effects.

Keywords. Canavanine; inhibition of growth; protein synthesis; heterocyst; differentiation; *Anabaena doliolum*.

Introduction

Several filamentous nitrogen-fixing cyanobacteria show a characteristic pattern of heterocysts oriented in a one-dimensional array. One way of investigating the formation of such a spaced pattern in an organism is to alter the pattern with certain chemicals. Some amino acid analogues such as 7-azatryptophan, L-methionine-DL-sulfoximine and rifamycin (an inhibitor of DNA-dependent RNA polymerase) significantly change the heterocyst pattern (Mitchison and Wilcox, 1973; Wolk, 1975; Singh *et al.*, 1977; Agrawal and Kumar, 1978; Ladha and Kumar, 1978). A logical assumption is that the production of some compound, possibly an inducer of heterocyst development, is blocked by these chemicals or by their metabolic products (Mitchison and Wilcox, 1973). However, very few chemicals are known to act in the reverse way (*i.e.*, blockage of the formation of an inducer of heterocyst development), except for a few specific protein synthesis inhibitors *e.g.*, chloramphenicol, actinomycin and combined nitrogen sources (the physiological inhibitor) which completely block heterocyst formation. In our study on amino acid analogues, we have found that canavanine (2-amino-4-guanidino-oxy-butan acid $NH_2C(=NH)$-NH-O-CH_2-$CH_3CH(NH_2)$) an analogue of arginine, strong inhibits growth and heterocyst differentiation in a blue-green alga *Anabae doliolum.* Canavanine causes an exponential inactivation of the bacteriu *Escherichia coli* under conditions of low intracellular arginine (Schachtele a Rogers, 1965). Since the growth inhibition by canavanine is often reversed arginine (Rosenthal, 1977), it has been suggested that canavanine interferes wi arginine biosynthesis and/or utilization. Arginine plays an important role in t synthesis of cyanophycin granules (Simon, 1973, 1976) and the polar nodules heterocysts in cyanobacteria and it was considered worthwhile to study the effe of canavanine on the various processes as no earlier study had been made. In t paper we describe the effect of canavanine on growth and heterocyst differentiati and the reversal of its toxicity by arginine and lysine, and compare these with t effects of a few other analogues.

Materials and methods

Organism and culture conditions

Anabaena doliolum was isolated from the local rice fields. The alga was ma unialgal and axenic adopting standard microbiological techniques and w routinely grown in Allen and Arnon's (1955) medium with or without combine nitrogen (10 mM KNO_3 and/or 2 mM NH_4Cl) at 27±2°C in a culture room. As th alga sporulated *en masse* at the end of the stationary phase of growth, a clon population was very easily raised from a single spore. The cultures we illuminated with cool white fluorescent tube lights at 2000 lux for 16 h daily. A experiments were performed with the cultures obtained at the log phase (6-d old). Growth experiments were carried out in culture tubes by measuring t absorbance in a Bausch and Lomb Spectronic 20 colorimeter at 665 nm. Heter cyst frequency was determined as % of total cells by counting at least 10 filamen (*ca* 600-1000 cells). Cyanophycin granules were observed microscopically an compared with those in untreated controls. The cytochemical test for cyanophyci granules was made by the method of Fuh (1968). Filaments were stained in saturated solution of carmine in 45% acetic acid for 10-15 minutes. Th cyanophycin granules developed a distinct red colour after staining. However, th presence of other granules was also taken into account. To obtain no heterocystous cultures, the cultures were previously grown in KNO_3 (0.02 M).

Isolation of canavanine-resistant mutant

Spores of *Anabaena doliolum* were collected by centrifugation at 3000 *g* for 10 mi washed at least 3-4 times and then incubated in 10 ml (Allen and Arnon's KNO medium for 48 h. This resulted in swelling of about 90% of the spores and th colour also became more deeply bluish-green. From this stage of spore suspension aliquots were withdrawn and treated with N-methyl-N'nitro-N-nitrosoguanidin (methylnitrosoguanidine) solution (100 µg/ml at pH 7) for 30 minutes (this trea ment permits about 50% survival). After 3-4 washings and centrifugations of th methylnitrosoguanidine-treated spore suspensions, 10^6 to 5×10^7 colony formin units were plated on *ca* 30 agar plates each having 10µg/ml canavanine. Then, the plates were transferred to the culture room and probable resistant clones arising after two weeks were picked up and tested for growth in canavanine supplemented plates as well as in a liquid culture medium. After 3-4 subcultures stable resistant clones were isolated and maintained in canavanine supplemented slants.

Chemicals

L-Canavanine, L-arginine, L-lysine, L-norleucine. α-methyl-DL-methionine, N-formyl-DL-methionine, α-methyl-DL-aspartic acid, L-methionine-DL-sulfoximine, DL-7-azatryptophan and chloramphenicol were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Methylnitrosoguanidine was kindly provided to us by Dr. C. Van Baalen of Marine Science Institute, USA, University of Texas. Other chemicals used were of the highest purity available from British Drug House (Glaxo), New Delhi. Solutions of all amino acids, amino acid analogues, chloramphenicol and methylnitrosoguanidine were sterilized by membrane filtration.

Results

Effect of canavanine on growth

Figure 1 shows that the addition of canavanine at a concentration as low as 1 µM results in inhibition of the algal growth. No growth occurred at concentrations of 10 µM or 100 µM of the chemical. Growth was not resumed even 12 days after the treatment. The transfer of the filaments treated for 48 h with 10 µM canavanine in

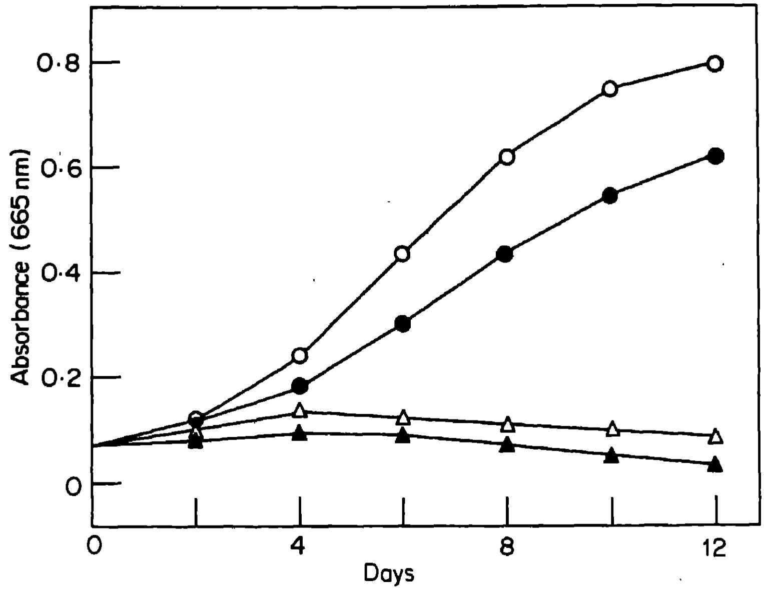

Figure 1. Effect of different concentrations of L-canavanine on growth of *Anabaena doliolum* in medium free of combined nitrogen. Control, (O); Canavanine, 1 µM (●); 10 µM (Δ); and 100 µM (▲).

an analogue-free medium also failed to elicit resumption of growth (data not Our results on growth inhibitions are in agreement with similar observation *coli* and *Chlamydomonas reinhardii* (Schachtele and Rogers, 1965; McM 1971). Microscopic examination of the canavanine-treated alga showed con loss of the characteristic granular inclusions, while such granules became frequent following treatment of the alga with chloramphenicol. This obser is based on the presence or loss of characteristic red coloured granular incl following acetocarmine staining of the filaments. Most probably, these gra represent the cyanophycin granules comprising of aspartic acid-arginin polymers.

Reversal of canavanine toxicity by arginine and lysine

The effect of canavanine (10 µM) is reversed by the simultaneous addition µM arginine (figure 2). There was no loss of granules from the stationary cultures as distinct red granules were observed in all the filaments of the cu after acetocarmine staining. Arginine alone stimulated growth and significantly repressed heterocyst frequency (figure 2 and table 1). There report of an arginine-induced stimulation of growth except in *Plecton boryanum* where arginine inhibited nitrogenase activity by acting as a nitr source (Nagatani and Haselkorn, 1978). Lysine did not relieve the canava induced inhibition of growth as efficiently as arginine (figure 2). There was poor growth with lysine + canavanine and this finding was in contrast to an ea study made on fungi, where lysine completely reversed inhibition of growt canavanine (Walker, 1955). Our finding is consistent with that of Aslam (1978), where arginine proved more effective than lysine in reversing canavar induced inhibition of the induction of nitrate reductase in corn roots. Lysine a did not show a significant effect either on growth or heterocyst frequency.

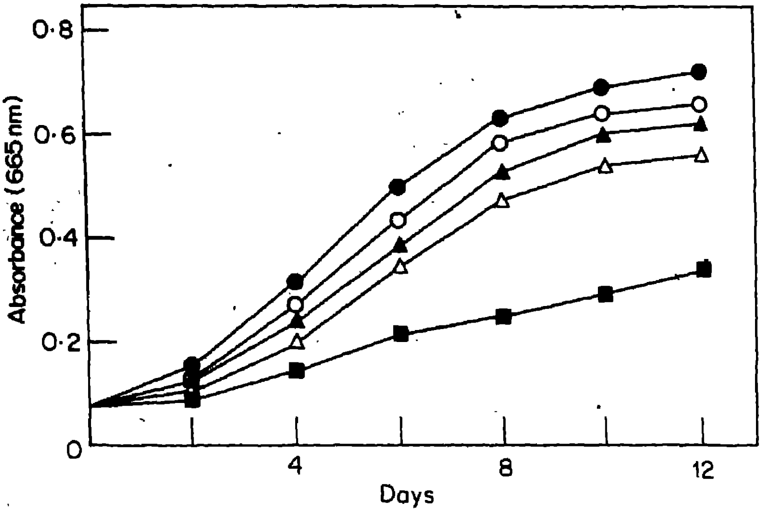

Figure 2. The effect of arginine and lysine on growth and on the reversal of L-canavanine induced toxicity. Control, (Δ); arginine 100 µM, (●); lysine 100 µM (▲); arginine 100 µM + canavanine 10 µM, (O); and lysine 100 µM + canavanine 10 µM, (■).

Table 1. Heterocyst frequency[a] in different concentrations of L-canavanine, arginine and lysine in a culture medium free of organic nitrogen compounds.

Addition (μM)	Proheterocysts %	Mature heterocysts %
None	2	4.5
L-canavanine (1.0)	0	0
L-canavanine (10.0)	0	0
L-lysine (100)	1.5	4.0
L-arginine (100)	1.5	2.5
L-arginine (100) + L-canavanine (10)	1.5	4.5
L-lysine (100) + L-canavanine (10)	1.5	3.0

[a] Heterocyst frequency was counted after 72 h, heterocysts having thick wall and developed polar nodules being counted as mature heterocysts.

Effect of canavanine on heterocyst production

Canavanine (1 μM or above) completely inhibited heterocyst formation (table 1). The simultaneous addition of arginine (100 μM) relieved the inhibition, and permitted the filaments to differentiate into heterocysts with a frequency comparable to that observed in the controls. However, there was only 3% heterocyst formation in a lysine + canavanine containing medium. This is consistent with the noted partial recovery of canavanine inhibited growth.

Effects of some other amino acid analogues on heterocyst differentiation

Amino acid analogues have been categorised into three groups *viz.*, group a, b and c on the basis of their effect on growth and heterocyst formation. Amino acid analogues of group (a) inhibited growth but had no effect on heterocyst frequency. In group (b), inhibition of growth was accompanied by a drastic change in heterocyst frequency and spacing. L-norleucine, α-methyl-DL-methionine, N-formyl-DL-methionine and α-methyl-DL-aspartic acid belong to this group. With 1 μM methionine sulphoximine, the heterocyst frequency increased to 8-9%, whereas with 100 μM 7-azatryptophan, the frequency was 13-14%. Finally the only compound in group (c), canavanine, inhibited growth as well as heterocyst formation. As shown earlier, 1 μM or higher concentrations of canavanine completely inhibited heterocyst formation. This shows that these effects of different amino acid analogues are similar.

Time-course study with canavanine and chloramphenicol on heterocyst differentiation

The effect of canavanine was compared with that of the known protein synthesis inhibitor, chloramphenicol in a short term experiment on heterocyst differentiation. The concentrations of canavanine (1 μM) and chloramphenicol (25 μg/ml) chosen were those which had been found to be nontoxic as judged by algal via survival after treatment with these concentrations and following the t treated cultures into drug-free medium. Canavanine or chloramphe added to non-heterocystous, nitrate-grown filaments at 0, 4, 8, 12 and incubationof these filaments in nitrogen-free medium. The addition o the chemicals to cultures in a heterocyst-inducing medium at 0, 4, and 8 complete suppression of heterocyst differentiation. Heterocyst formatio suppressed when the chemicals were added at 12 or 24 h. This observatio that canavanine blocked the formation of proteins, some of which required for heterocyst differentiation and which may be formed w following transfer of a non-heterocystous culture to a heterocyst i medium.

Canavanine-resistant mutant

The canavanine resistance mutation frequency following mutagenesis us soguanidine was much higher than the frequency of spontaneous mutation The canavanine-resistant mutant strain showed a slightly faster gro than the parent strain. The heterocyst frequency also increased from 4. in the mutant. However, the filaments were highly granulated in contrast t of granules in the canavanine-treated wild type. Further the mutant require arginine for normal growth, but showed better growth with ca than without it. The mutant alga showed resistance to canavanine even growth in a canavanine-free medium for a long time.

Table 2. Mutation frequency, generation time[a] and heterocyst frequency of pare Cnv^R (L-canavanine resistant mutant)

Strain and growth condition	Mutation frequency		Generation time (h)	Heterocyst frequency (%)
	spontaneous	Induced (by NTG)		
Parent (combined nitrogen-free medium)	–	–	50	4.5
Cnv^R (with L-canavanine, 10 ug/ml)	1.52×10^{-7}	5.63×10^{-4}	46	5.5
Cnv^R (Without L-canavanine)	–	–	52	5.0

[a] Values given are average of triplicates and represent the time taken by the growing culture to double its original absorbance in the exponential phase of growth; time was de mined directly from the growth curve.

1 μM canavanine=0.17 μg/ml.

Discussion

The above data indicates the following tentative conclusions concerning the effect of canavanine.

Firstly, the action of canavanine is quite different from that of the other amino acid analogues discussed above. It was highly toxic even at very low concentrations. The observed reversal of toxicity by the simultaneous addition of arginine favoured the competitive utilization of canavanine by the alga. Arginine was more effective than lysine in reversing the canavanine-induced inhibition of growth and heterocyst differentiation. This is consistent with earlier observations of the effects of canavanine on the growth of maize embryos and on the induction of nitrate reductase in corn roots (Rosenthal, 1977; Aslam *et al.*, 1978). The paucity of cyanophycin granules in canavanine-treated material supports the findings of Simon (1976), who showed that the incorporation of (^{3}H)-labelled arginine by multi-L-arginyl-(poly) aspartic acid synthetase was insensitive to the addition of a variety of antibiotics including chloramphenicol, erythromycin, streptomycin, tetracycline, rifamycin, 7-methyltryoptophan and 7-azatryptophan, However, the addition of canavanine inhibited (upto 72%) the incorporation of (^{3}H)-arginine and the inhibition could be overcome by increasing the concentration of L-arginine in the reaction mixture. Chloramphenicol-induced formation of cyanophycin granules in this alga confirms the earlier reports in *Agmenelum quadruplicatum* and *Anabaena cylindrica* (Ingram *et al.*, 1972; Simon, 1973).

Secondly, the inhibition of heterocyst differentiation by canavanine is unique in the context of the known effects of other amino acid analogues. In the case of L-methionine-DL-sulfoximine it is known that glutamine synthetase is inhibited and it leads to a decrease in the intracellular level of glutamine and excretion of ammonia into the medium resulting in changes in the frequency and spacing of heterocysts (Stewart and Rowell, 1975; Ownby, 1977; Singh *et al.*, 1977; Ladha and Kumar, 1978). On the other hand 7-azatryptophan was incorporated, in the place of tryptophan resulting in the formation of defective proteins. Mitchison and Wilcox (1973) proposed that this defective protein was solely responsible for the observed alteration in heterocyst pattern formation. No previous report on the influence of canavanine on the heterocysts is available. However, the action of canavanine as an arginine analogue has been summarized in the recent reviews (Norris and Lea, 1976; Rosenthal, 1977). It has been well established that heterocyst differentiation is dependent on the synthesis of a nascent protein and also on a product of the photosynthetic process (Singh and Srivastava, 1968; Singh and Kumar, 1971; Tyagi, 1975; Haselkorn, 1978). Chemicals affecting either of the two processes inhibited heterocyst differentiation (Tyagi, 1975). From our study, the effect of canavanine appeared to be primarily on the synthesis of proteins. Possibly, canavanine inhibited protein synthesis by acting as a substrate for arginyl-tRNA. The inhibition of protein synthesis in a heterocyst-inducing medium by canavanine resulted in the inhibition of heterocyst differentiation. In this case preformed cellular proteins may be insufficient to fulfil the complete manifestation of heterocyst differentiation. This inhibition of protein synthesis was not stage-specific.

This is indicated by the data of the time-course experiment where even after (the time for proheterocyst formation), the maturation of heterocysts was blocl However, in addition to the direct inhibition of nascent protein synth canavanine might also be directly incorporated into polypeptides resulting in formation of a defective protein(s). Such defective proteins could be the caus inhibition of growth and also heterocyst differentiation. Formation of defec canavanyl protein(s) has been shown in *E. coli* by Sachachtele *et al.* (1968).

The mechanism of canavanine resistance in the mutant strain is not known possible cause of resistance might be a defective arginyl-tRNA synthetase in mutant. Similar mutants resistant to canavanine were previously isolated bacteria and *Chlamydomonas* (Sachachtele *et al.*, 1968; McMahon, 1971).

The mutant is interesting in view of the generally observed mutational immur of *A. doliolum* to certain resistance markers, e.g., antibiotic resistance (Ladha Kumar, 1978). There is only one previous report of an ethionine-resistant mut of a non-nitrogen fixing alga *Plectonema boryanum* (Hentschel *et al.*, 1978) a hence the isolation of this mutant should greatly advance our understanding biosynthesis and regulation of amino acids. This mutant was stable even af several subcultures and canavanine-free medium and thus seems to offer a suital genetic marker in the absence of other antibiotic markers in this alga.

Acknowledgements

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Excretion of lysine by *Micrococcus glutamicus*

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Abstract. Analysis of intracellular and extracellular lysine concentration during lysine fermentation by *Micrococcus glutamicus* AEC RN-13-6/1 indicated that lysine excretion occurs against a concentration gradient towards the end of the fermentation period. The capacity to excrete lysine against a concentration gradient may be a factor contributing to the high yield of lysine.

Keywords. *Micrococcus glutamicus*; lysine production.

Introduction

Analysis of intracellular and extracellular amino acids in biotin-sufficient and biotin-deficient cells of *Micrococcus glutamicus* indicated that a deficiency of biotin stimulated the synthesis of glutamate and the excretion of amino acids (Kimura, 1963). Similar analyses indicated that cells of *Brevibacterium flavum* release intracellular amino acids into wash solutions more readily when grown under biotin-deficient conditions (Shiio *et al.*, 1962).

This paper describes investigations on lysine excretion by a lysine over-producing strain of *M. glutamicus* by measurement of the concentration of free lysine and of the total free amino acids inside and outside the cells during lysine fermentation.

Materials and methods

All amino acids and their derivatives (except threonine which was used in growth media), dried cells of *Bacillus cadaveris* (used as a source of lysine decarboxylase), Dowex ion-exchanger and Blue Dextran B-2000 were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. L-threonine used in the growth media was a product of Kyowa Hakko Kogyo Limited, Tokyo, Japan. Polyvinyl alcohol (Cellacell grade 198) was from Cellulose Products of India Limited, Ahmedabad. All other chemicals were of the highest purity available.

M. glutamicus AEC RN-13-6/1 was the gift of Dr V.C. Vora, Central D
Research Institute, Lucknow; this is a homoserine-requiring, S-(2-aminoethyl) cysteine (AEC)-resistant mutant of *M. glutamicus* ATCC 13059 and was maintain
on slants of medium B (Sano and Shiio, 1967) containing S-(2-aminoethyl) cysteine. HCl (2 mg/ml), L-threonine (2 mg/ml) and DL-methionine (50 µg/n
Cells were grown in a medium containing clarified corn steep liquor (Lakshm
1980). Cells in the log phase obtained by two transfers into clarified corn ste
liquor medium were used as inoculum (10%, v/v).

Viable counts were made on nutrient agar plates after appropriate dilution of t
sample with 0.9% saline. Total bacterial counts were made in a haemocytome
with 0.4% polyvinyl alcohol in 0.9% saline containing 0.5% formalin as t
suspending medium. Samples were diluted as required with 0.9% saline contain
0.5% formalin. Individual cells in a cluster were mounted.

The volume of a pellet of packed cells is the sum of the volume occupied by t
cells (or cell volume) and the intercellular volume. The intracellular volume a
the volume of a pellet of packed cells were measured essentially as described
Roberts *et al.* (1955). Cells were packed by centrifugation at 27,000 *g* for 20 m
The concentration of Blue Dextran-2000 in solution was measured by
absorbance at 630 nm. The cell volume, calculated by difference, was correlat
to cell mass and number as described by Roberts *et al.* (1955) except that total c
counts were made.

For the extraction of intracellular amino acids, wet-packed cells from 48 ml
broth were washed (twice) rapidly with 20% sucrose (20 ml). The washed ce
were extracted repeatedly, by shaking with aliquots (30 ml) of 70% ethanol for
min at 270 rpm, until a 100 µl aliquot was ninhydrin-negative. The pool
extracts were evaporated to dryness *in vacuo* at 50°C. They were then taken up
10 ml water and desalted on a column (1×5 cm) of Dowex 50-H$^+$ (X8, 200-4
mesh). The resin was washed with water and the amino acids eluted with 2
NH_4OH. The ammoniacal effluent was dried *in vacuo* at 60°C. It was taken up
a known volume of water. Lysine was separated from other amino acids by hi
voltage electrophoresis at 2500 V on Whatman No. 3 MM filter paper (28×60 c
in acetic acid-formic acid buffer pH 1.8 in a Savant apparatus (Blackburn, 196
After electrophoresis, the paper was dried at 80°C for 60 min. Lysine w
quantitated using ninhydrin-cadmium acetate reagent (Blackburn, 1965). To
amino acids were estimated against standard leucine by the method of Spies (195
Lysine was also identified by decarboxylation by using the lysine decarboxylase
Bacillus cadaveris (Dickerman and Carter, 1962).

The culture filtrate was deproteinized by the addition of 0.5 volume of 50
perchloric acid. Amino acids in 1.5 ml of deproteinized culture filtrate we
desalted on a column (1.4×5.4 cm) of Dowex 50-H$^+$. Lysine and total amino aci
were estimated as above.

Results and discussion

The growth pattern of *M. glutamicus* is shown in figure 1.

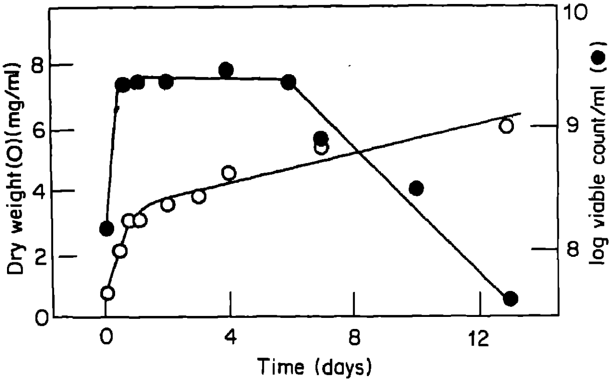

Figure 1. Growth pattern of *M. glutamicus* AEC RN-13-6/1

On an average it was found that 1 mg dry weight of 24 h cells corresponded to 4.45 mg wet weight, 0.52 µl cell volume and 1.07×10^9 cells (or 0.86×10^9 viable cells).

The intracellular and extracellular concentrations of lysine and total amino acids during the fermentation period are shown in table 1. Comparison of the data in figure 1 and table 1 shows that the viable counts increased rapidly during the first 24 h and remain constant for around 7 days. During this time, the cells synthesize lysine, most of which accumulates in the medium. The inference that most of the lysine arises by biosynthesis is based on the known amino acid composition of corn steep liquor (Cardinal and Heedrick, 1948; Aurich, 1960), on the basis that the corn steep liquor medium initially contained only 2 µmol lysine per ml (unpublished data) and because the specific activities of aspartokinase, dihydrodipicolinate synthetase and diaminopimelate decarboxylase differed only slightly (10% increase, 22% decrease and 22% increase respectively) between the 16th and 144th hour of fermentation (unpublished data).

Although the intracellular concentration of lysine is greater than the extracellular concentration in the initial stages of fermentation, it is less towards the end of the fermentation period (table 1). Thus, lysine is excreted against a concentration gradient towards the end of the fermentation period. Further support to this conclusion is lent by the observation that lysine is accumulated within the cells after viable counts start falling off (figure 1, table 1).

It may be assumed that in cells which can no longer divide, the biosynthetic and energy yielding machinery have become less organised. The viability of several bacteria is reported to decrease when the adenylate charge in the cells decreases to values below 0.8 (Chapman and Atkinson, 1977). The physiological changes occurring in cells that cannot divide would contribute significantly to those observed in the 'death phase'. It has been established that bacteria accumulate amino acids in cells to levels several times higher than those in the growth medium (Harold, 1972). Efflux of intracellular amino acids occurs upon inhibition of energy-generating mechanisms either by the use of uncouplers, by the inhibition of

Table 1. Intracellular and extracelluar concentrations of amino acids during lysine fermen tion.

Time (h)	Intracellular			Extracellular		
	Lysine [a] (nmol/mg dry weight)	Total amino acids (nmol/mg dry weight)	Mole fraction of lysine	Lysine [b] (μmol/ml or mM)	Total amino acids (μmol/ml)	Mol fractio lysin
0	1 (2)	150	0.007	2	18	0.11
8	5 (10)	180	0.028	3	18	0.17
16	19 (37)	590	0.032	5	22	0.23
24	26 (52)	650	0.40	13	23	0.57
48	16 (31)	460	0.035	31	57	0.54
72	19 (37)	430	0.044	37	69	0.54
96	21 (40)	370	0.056	54	90	0.60
168	15 (29)	250	0.060	83	93	0.89
240	28 (54)	180	0.155	94	144	0.65
312	40 (77)	160	0.250	81	148	0.55

Values in parenthesis represent intracellular concentration (mM i.e., μmol/ml cell volume). 1 m weight of cells was taken to be 0.52 μl cell volume.

[a] The estimated lysine was completely decarboxylated by lysine decarboxylase (*B. cadaveris*, S Chemical Co., USA).

[b] Intracellular and extracellular lysine concentration as estimated by ion-exchange chromatog (Durrum Microbore Fluoropa Automatic Analyzer, Kit MBF, Durrum Chemical Co., USA) at t 72 and 168th h of fermentation agreed with the electrophroetec value to within 5%.

electron transport or by the starvation of organisms such as *Streptococcus la* which have no energy reserves (Brown, 1971; Kessel and Lubin, 1962; Thomas Batt, 1969). Therefore, increase in the intracellular lysine concentration in phase of logarithmic decrease in viable counts suggests that the concentratio lysine outside the cell was maintained at a higher level than the intracellular ly concentration, at the expense of cellular energy, before the viable counts sta falling off.

Copious excretion of primary metabolites such as citric acid, amino acid nucleoside bases by microorganisms is well documented (Rose, 1978). extracellular concentration attained in these fermentation are very high. It is known whether excretion occurs against a concentration gradient in these fern tations also. There is a report of 'active' excretion of lysine occuring in an A resistant mutant of *Brevibacterium lactofermentum* (Nakayama, 1976). details of the experiments leading to this inference are not available.

The possible advantage in having a transport system capable of excreting amino acid to levels higher than those found within the cell is that excretion continue uninterrupted to any extent, while the intracellular concentratior maintained at low, presumably 'non-toxic' levels, levels low enough to preven cessation of its synthesis or not high enough to stimulate its breakdown.

Further experimentation is required to show whether the cells of *M. glutamicu* have active lysine uptake systems functioning in the early stages of fermentatio (table 1) which start functioning in the direction of active exit or whether the cell have a transport system for lysine in which coupling to cellular energy occurs in th reverse direction, so that it is able to establish a concentration gradient with highe concentration outside.

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Aspartokinase of a lysine producing mutant of *Micrococcus glutamicus*

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Abstract. Aspartokinase from *Micrococcus glutamicus* AEC RN-13-6/1 [a homoserin requiring, S-(2-aminoethyl)-L-cysteine resistant, lysine producing strain] was purified 71 fold The partially purified enzyme was inhibited by L-lysine. L-threonine, L-methionine, L isoleucine, L-valine and L-phenylalanine activated the enzyme and reversed the inhibition by L lysine. Aspartokinase activity was not derepressed by growth-limiting concentrations of L threonine and/or L-methionine. It was not repressed by an excess of L-lysine (20 mM) and/o L-isoleucine (15.3 mM). The degree of activation or inhibition by amino acids was dependan on the composition of the growth medium. This observation is in contrast with the enzym from the original (non-lysine-producing) strain which was inhibited by lysine or threonine and in a concerted manner by threonine plus lysine.

Keywords. Aspartokinase; *Micrococcus glutamicus*.

Introduction

The properties of aspartokinases (adenosine triphosphate (ATP): L-aspartate-4-phosphotransferase, EC 2.7.2.4) of different strains of *Micrococcus glutamicu* have been investigated only in crude extracts of cells (Kase and Nakayama, 1974; Nakayama *et al.*, 1966; Zaitseva and Chernysheva, 1975). Amino acid auxotroph of *M. glutamicus* were found to have aspartokinase activities sensitive t concerted/synergistic feedback inhibition by lysine plus threonine, although the response of the enzyme to lysine or threonine alone differed in these various strains (Kase and Nakayama, 1974; Nakayama *et al.*, 1966; Zaitseva and Chernysheva, 1975). Screening for resistance to S-(2-aminoethyl)-L-cysteine (AEC), a lysine analogue, in an organism with aspartokinase activity sensitive to concerted/synergistic feedback inhibition by lysine plus threonine yielded mutants with a variety of feedback inhibition patterns (Hermann *et al.*, 1972; Kase and Nakayama, 1974; Shiio *et al.*, 1970; Tosaka *et al.*, 1978). Repression of the aspartokinase activity of *M. glutamicus* has not been previously investigated.

Abbreviations used: AEC or aminoethyl cysteine, S-(2-aminoethyl)-L-cysteine; Lys, L-lysine; L-Asp, L-aspartate; Thr, L-threonine; Ile, L-isoleucine.

The partial purification, properties and repression of aspartokinase from *M. glutamicus* AEC RN-13-6/1 (homoserine$^-$, AEC^R; a lysine excreting strain) are reported in this paper. The feedback inhibition properties of the aspartokinase of this organism differ from those of the AEC^R mutants described earlier (Kase and Nakayama, 1974).

Materials and methods

Chemicals

DL-aspartic-β-semialdehyde was prepared by ozonolysis of DL-C-allylglycine (Black and Wright, 1955). Alumina type 305, DEAE-Sephadex, ATP and all amino acids were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. L-threonine used in growth media was a product of Kyowa Hakko Kogyo Limited, Tokyo, Japan. All other chemicals were of the highest purity available.

Bacteria and growth

M. glutamicus AEC RN-13-6/1 was a gift from Dr V. C. Vora, Central Drug Research Institute, Lucknow. It was obtained from *M. glutamicus* No. 267 (homoserine$^-$) by ultraviolet (UV) mutagenesis and selection on plates with medium B (Sano and Shiio, 1967) containing aminoethyl cysteine (2 mg/ml) and L-threonine (2 mg/ml). *M. glutamicus* No. 267 was itself derived from *M. glutamicus* ATCC 13059 by UV mutagenesis (V. C. Vora, personal communication). *M. glutamicus* AEC RN-13-6/1 excreted 16 g/1 lysine HCl under favourable growth conditions. Under identical growth conditions, the lysine HCl content of the culture filtrate of *M. glutamicus* ATCC 13059 did not rise above the 60 mg/ml level observed in uninoculated media (Lakshman, 1980).

M. glutamicus AEC RN-13-6/1 was maintained on slants of medium B (Sano and Shiio, 1967) containing aminoethyl cysteine (2 mg/ml), L-threonine (2 mg/ml) and DL-methionine (50 µg/ml). Medium B contained ammonium sulphate, 1.5 g; urea, 1.5 g; KH_2PO_4, 1 g; K_2HPO_4, 3 g; $MgSO_4.7H_2O$, 0.1 g; $CaCl_2.2H_2O$, 0.001 g; *d*-(+)-biotin, 30 µg; thiamine-HCl, 100 µg; trace element solution, 1 ml and distilled water to a final volume of 1000 ml. The pH was adjusted to 7.0 before sterilization. Glucose was sterilized separately as a 25% solution and added to the medium to 0.5%. The trace element solution contained $ZnSO_4.7H_2O$, 8.8 g; $FeCl_3.6H_2O$, 0.97 g; $CuSO_4.5H_2O$, 0.393 g; $Na_2B_4O_7.10H_2O$, 88 mg and distilled water, 1000 ml. Cells were grown with shaking (270 rpm) at 30° C, in one-litre Erlenmeyer flasks containing 200 ml medium B suplemented with L-threonine (2 mg/ml) and DL-methionine (50 µg/ml). They were harvested in the late exponential phase by centrifugation (10,000 *g*, 20 min), washed twice with 0.9% NaCl at 4°C and stored frozen as a pellet.

For repression experiments, cells in the exponential phase of growth in medium B containing L-threonine (2 mg/ml) and DL-methionine (50 µg/ml), were washed once with sterile medium B (glucose omitted), suspended in sterile medium B to the original density and inoculated into medium B with various amino acid suplements.

The cells were harvested towards the end of the exponential phase of growth an processed as above.

Medium C (V. C. Vora, personal communication), a growth medium favourabl for lysine production, contained glucose, 15 g; NH_4Cl, 1 g; $MgSO_4.7H_2O$, 0.02g corn steep liquor, 5 g; urea, 0.25 g; K_2HPO_4, 0.10 g and distilled water, 1000 ml Corn steep liquor, urea and K_2HPO_4 were autoclaved separately. Corn stee liquor was clarified before use as described elsewhere (Lakshman, 1980). Th various components were mixed and the pH adjusted to 7.0 just before inoculation.

Enzyme assays

Aspartokinase was assayed by estimating the aspartate-β-hydroxamate forme from β-aspartyl phosphate (Black, 1962). The reaction mixture contained in 0. ml, aspartate-Tris (pH 7.5), 50 µmol; ATP (pH 7.5), 10 µmol; $MgCl_2$, 10 µmol $NH_2OH.KCl$ (pH 7.5) 320 µmol and enzyme. The reaction was allowed to proceed for 40 min at 37°C, and terminated by the addition of 0.75 ml of ferric chloride reagent (10% ferric chloride.$6H_2O$ and 3.3% trichloroacetic acid in 0.7 N HCl). The precipitated protein were removed by centrifugation and the absorbance o the supernatant solution was measured at 540 nm in a Beckman spectrophotomete model DU-2. A unit of enzyme activity catalyzes the formation of 1 µmol o aspartate-β-hydroxamate per min at 37°C.

Dihydrodipicolinate synthetase [L-aspartate-β-semialdehyde hydrolyase (adding pyruvate and cyclizing) EC 4.2.1.52] was assayed by measuring the change in absorbance at 270 nm in a Beckman spectrophotometer model DU-2, in the presence of imidazole (Yugari and Gilvarg, 1965). The assay system contained per ml, imidazole-HCl buffer (pH 7.4), 100 µmol; sodium pyruvate, 15 µmol; DL-aspartic-β-semialdehyde, 1 µmol and the enzyme. A unit of enzyme activity has been defined as the amount of enzyme producing an increase in absorbance of 1.00 per min at 270 nm (10 min after addition of all components). Diaminopimelate decarboxylase (*meso*-2, 6-diaminopimelate carboxylase, EC 4.1.1.20) was assayed by measuring the rate of diaminopimelate disappearance (White, 1971). The assay system contained per ml, Tris-HCl buffer (pH 7.5), 100 µmol, diaminopimelate, 5 µmol, pyridoxalphosphate 50 nmol and enzyme. The reaction was started by the addition of the substrate and incubation was for 20 min at 37°C. At zero time and after 20 min, 0.1 ml of the reaction mixture was withdrawn into tubes containing 0.4 ml water plus 0.4 ml glacial acetic acid. 0.5 ml of the ninhydrin reagent (250 mg ninhydrin in 6 ml glacial acetic acid and 4 ml 0.6 M phosphoric acid) was added. The colour was developed at 37°C for 90 min and measured at 440 nm as for aspartokinase. A unit of enzyme activity catalyzes the disappearance of 1 µmol of diaminopimelate per min at 37°C.

Analytical methods

Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Results

Purification of aspartokinase

All the purification steps were carried out at 4-8°C. Frozen *M. glutamicus* cells were thawed, ground with three times their weight of alumina and extracted with 0.1 M potassium phosphate buffer (pH 7.0) containing 20 mM 2-mercaptoethanol, 1 mM L-lysine and 1 mM L-threonine (phosphate-mercaptoethanol-Lys-Thr buffer 5 ml/g wet-packed cells). The resulting suspension was centrifuged at 10,000 *g* in a Sorvall RC-2B centrifuge for 10 min and the clear supernatant termed as crude extract. The crude extract was centrifuged at 103,000 *g* for 60 min in a Beckman Model L2-65B ultracentrifuge. A 25% (w/v) solution of streptomycin sulphate in posphate-mercaptoethanol-Lys-Thr buffer was added slowly to the clear 103,000 *g* supernatant to give a final streptomycin sulphate concentration of 3%. Material which precipitated on standing overnight was removed by centrifugation at 27,000 *g* for 20 min. The supernatant was fractionated with solid ammonium sulphate at pH 7.0. After keeping for 60 min, the precipitated proteins were collected by centrifugation at 27,000 *g* for 20 min. Proteins precipitating between 50 and 60% saturation with ammonium sulphate were taken up in phosphate-mercaptoethanol-Lys-Thr buffer and dialyzed against the same buffer for 3 h with rapid stirring and change of buffer every 30 min. The dialyzed enzyme was loaded

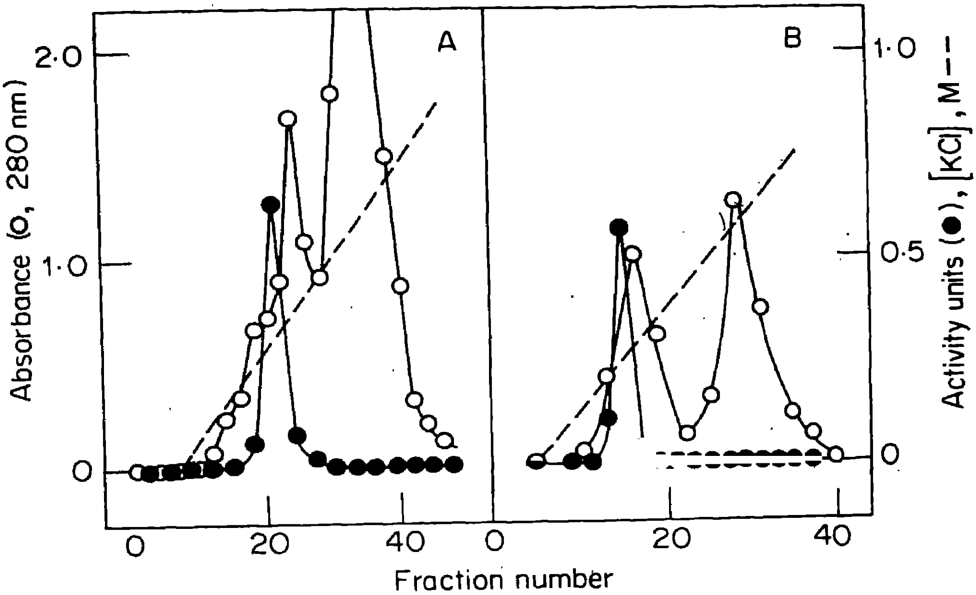

Figure 1. DEAE-Sephadex chromatography of aspartokinase,

A. Fraction 3 (8 ml) (table 1) was loaded on a column (1×23 cm) of DEAE-Sephadex A-50 equilibrated with phosphate-mercaptoethanol-Lys-Thr buffer. The two chambers of the gradient apparatus contained 75 ml of the above buffer and buffer containing 1 M KCl, respectively. Fractions (3.2 ml) were collected at 5 min intervals.

B. Fraction 4 (20 ml) (table 1) was chromatographed under the conditions described above except that phosphate-mercaptoethanol-isoleucine buffer was used instead of the buffer containing Lys and Thr. Fractions (2.2 ml) were collected at 5 min intervals.

on a column (1×23 cm) of DEAE-Sephadex equilibrated with phosphate r ethanol-Lys-Thr buffer. The column was washed with the same buffer absorbance at 280 nm was negligible. A linear gradient of KCl (0 to 1 same buffer was then applied. Fractions containing aspartokinase acti pooled, dialyzed as described above against 0.1 M potassium phosphate b 7.0) containing 20 mM 2-mercaptoethanol and 1 mM L-isoleucine and recl graphed on DEAE-Sephadex (figure 1). A summary of the purification p is given in table 1. The resultant enzyme preparation was not homoge evidenced by the electrophoretic pattern on 7.5% polyacrylamide gels a Further purification was not possible, although other procedures we Chromatography on Sephadex G-200 and Sepharose-4B (and its der caused inactivation of the enzyme.

Table 1. Partial purification of aspartokinase from *M. glutamicus* AEC RN-13-6/

Fraction	Total protein (mg)	Total activity (units)	Specific activity m units/mg protein)
10,000 *g* supernatant	638 [a]	10.9	17
103,000 *g* supernatant	434	11.4	26
50-60% saturation ammonium sulphate precipitate	91	5.2	57
DEAE-Sephadex I Chromatography	7	4.3	640
DEAE-Sephadex II Chromatography	3.1	3.8	1212

[a] From 18 *g* wet-packed cells

Properties of aspartokinase

Aspartokinase activity was stabilized by inclusion of L-lysine, L-lysine threonine, L-isoleucine or L-aspartate in the buffers used for extraction or The enzyme activity was completley lost within five days at 4°C in 0.1 M po phosphate buffer (pH 7.0) containing 20 mM 2-mercaptoethanol. The enz 50% activity in three weeks in phosphate-mercaptoethanol-Lys-Thr buffer phate-mercaptoethanol-Ile buffer at 4°C; 50% activity was lost in two weeks potassium phosphate buffer (pH 7.0) containing 20 mM 2-mercaptoethan mM L-aspartate at 4°C. The crude enzyme was optimally active between and 7.5. Mg^{2+} ions were required for activity. KCl was not required for and added KCl upto 640 mM had no effect. The enzyme was markedly stir by ammonium sulphate (150 mM), but K_2SO_4, Na_2SO_4, NH_4Cl and NaCl at t concentration had no effect.

The aspartokinase activity of *M. glutamicus* AEC RN-13-6/1 in partially purified preparations was inhibited by L-lysine. L-threonine, L-methionine and L-isoleucine stimulated the activity and reversed the L-lysine inhibition (table 2). In addition, L-valine or L-phenylalanine also stimulated the activity and reversed the inhibition by L-lysine. L-valine was the most effective amino acid in reversing the inhibition by L-lysine (data not shown). The effects of L-lysine, L-threonine and L-lysine plus L-threonine were the same in the presence and absence of ammonium sulphate (150 mM).

Table 2. Effect of amino acids on the aspartokinase activity of *M. glutamicus* AEC RN-13-6/1.

Additions (10 mM)	Per cent activity			
	Partially purified enzyme [a]	Crude enzyme [b]		
		Growth medium 2	Growth medium 7	Growth medium C
None	100	100	100	100
L-Lys	27	39	29	40
L-Thr	150	313	242	137
L-Met	173	344	240	167
L-Ile	183	352	266	179
L-Lys + L-Thr	59	124	92	69
L-Lys + L-Met	72	157	109	68
L-Lys + L-Ile	100	216	157	95
L-Lys + L-Thr + L-Met + L-Ile	96	ND	ND	96
L-Thr + L-Met	ND	344	220	166

[a] Fraction 4, table 1 was used as the enzyme.

[b] Crude extracts of cells were prepared in phosphate-mercaptoethanol-asparate buffer and dialyzed against the same buffer before use.

Composition of growth media 2 and 7 are shown in table 3.

ND—Not determined.

Repression of aspartokinase

Aspartokinase was not derepressed by growing cells with limiting concentration of L-threonine and/or L-methionine (table 3). Added L-lysine (20 mM) and/or L isoleucine (15.3 mM) had no significant effect on the levels of the enzyme. Th enzyme was significantly repressed in medium C.

Table 3. Effect of growth medium composition on the levels of the aspartokinase activity of *M. glutamicus* AEC RN-13-6/1.

Medium	Supplements to growth medium	Relative specific activity (%)
B	L-Thr (2 mg/ml) + L-Met (2 mg/ml)	100 (18[a])
	L-Thr (60 μg/ml) + L-Met (10 μg/ml)	71
	L-Thr (2 mg/ml) + L-Met (10 μg/ml)	74
	L-Thr (60 μg/ml) + L-Met (2 mg/ml)	74
	L-Thr (2 mg/ml) + L-Met (2 mg/ml) + L-Lys (3 mg/ml)	106
	L-Thr (2 mg/ml) + L-Met (2 mg/ml) + L-Ile (2 mg/ml)	93
	L-Thr (2 mg/ml) + L-Met (2 mg/ml) + L-Ile (2 mg/ml) + L-Lys (3 mg/ml)	97
C[b]		31

[a] Specific activity in m units per mg protein.

[b] Medium C has been described in Materials and methods. Cells were harvested in the late exponential phase.

The enzyme was assayed in dialyzed crude extracts. Similar values, less marked, were obtained with undialyzed crude extracts. Phosphate-mercaptoethanol-aspartate buffer was used for extraction and dialysis.

Total activity paralleled cell yield: 2.5-4.0 g/l in media 2-4; 7.0-9.0 g/l in media 1 and 5-7 and 17-22 g/l in medium C.

Aspartokinase activities of cells grown in media 2, 7 and C responded to feedback modifiers essentially like the partially purified enzyme (table 2). The enzyme from cells grown in media 2 and 7 was activated to greater extents by L-threonine, L-methionine or L-isoleucine; reversal of L-lysine inhibition by these amino acids was correspondingly greater. The response to feedback modifiers was the same in the exponential phase and the mid and late stationary phases of growth.

Dihydrodipicolinate synthetase and diaminopimelate decarboxylase

Dihydrodipicolinate synthetase and diaminopimelate decarboxylase of *M. glutamicus* AEC RN-13-6/1 were not repressed by an excess of L-threonine, L-methionine, L-lysine or L-isoleucine in the growth medium (table 4). Dihydrodipicolinate synthetase was insensitive to the amino acids of the aspartate family, added singly or in combinations at 10 mM, whereas diaminopimelate decarboxylase was inhibited by L-lysine and to a greater extent by a combination of lysine, threonine, methionine and isoleucine (table 5).

Table 4. Effect of composition of growth medium on levels of dihydrodipicolinate synthetase and diaminopimelate decarboxylase of *M. glutamicus* AEC RN-13-6/1.

Composition of media	Relative specific activity (%)	
	Dihydrodipicolinate synthetase	Diaminopimelate decarboxylase
Medium B + L-Thr (2 mg/ml) + L-Met (2 mg/ml)	100 (0.110[a])	100 (0.018[a])
Medium B + L-Thr (60 µg/ml) + L-Met (10 µg/ml)	90	71
Medium B + L-thr (2 mg/ml) + L-Met (10 µg/ml)	88	117
Medium B + L-Thr (60 µg/ml) + L-Met (2 mg/ml)	89	67
Medium B + L-Thr (2 mg/ml) + L-Met (2 mg/ml) + L-Lys (3 mg/ml)	96	116
Medium B + L-Thr (2 mg/ml) + L-Met (2 mg/ml) + L-Ile (2 mg/ml)	100	100
Medium C	64	139

[a] Specific activity in units per mg protein (Dl +*meso*)-diaminopimelate containing 53% of the *meso*-isomer was used for assay.

Crude extracts, dialyzed against 0.1 M potassium phosphate buffer (pH 7.5) containing 20 mM 2-mercaptoethanol, were used for assay.

Total activity paralleled cell yield (table 3).

Table 5. Effect of amino acids on dihydrodipicolinate synthetase and diaminopimelate decarboxylase activities.

Additions to reaction mixture (10 mM each)	Relative specific activity (%)	
	dihydrodipicolinate synthetase	Diaminopimelate decarboxylase
None	100 (0.110[a])	100 (0.018[a])
L-Lys	100	43
L-Thr	98	94
L-Met	112	94
L-Ile	98	100
L-Lys + L-Thr + L-Met + L-Ile	98	25

[a] Specific activity in units per mg protein.

Discussion

Selection for resistance to aminoethyl cysteine yields mutants either w derepressed levels of aspartokinase (Hermann *et al.*, 1972), altered sensitivity aspartokinase to feedback modifiers (Hermann *et al.*, 1972; Jegede *et al.*, 19 Shiio *et al.*, 1970; Tosaka *et al.*, 1978), altered sensitivity of dihydrodipicolin synthetase to feedback inhibitors (Hermann *et al.*, 1972) or altered lysine transp systems (Halsall, 1975).

The aspartokinase activity of *M. glutamicus* ATCC 13059, the parent strain the mutant used here, was inhibited by L-lysine, slightly inhibited or activated by threonine depending on the growth medium and subjected to concerted feedba inhibition by L-lysine plus L-threonine (table 6). The enzyme was stimulated by isoleucine, L-valine, L-methionine and L-phenylalanine; these amino acids revers the multivalent inhibition by L-lysine plus L-threonine (unpublished data). T enzyme therefore resembled the aspartokinase of *Brevibacterium flavum* No. 22 (Shiio and Miyajima, 1969). The aspartokinase activity of the AEC^R muta

Table 6. Effect of amino acids on the aspartokinase activity in crude extracts of cells of different *M. glutamicus* strains.

Strain	Per cent activity in the presence of (mM)							Effect of L-Ile on multivalent inhibition by L-Lys+L-Thr	Reference
	L-Lys		L-Thr		L-Lys+ L-Thr		L-Ile		
	1	10	1	10	1 each	10 each	10		
ATCC 13059	85	40	96	82	32	19	173	Reverses	Lakshman, 198
AEC RN-13-6/1	86	40	134	137	118	69	179	Reverses	Lakshman, 198
901 (homoserine)		94		123		52	123	Nil	Nakayama *et al* 1966
KY 9159 (Met$^-$)	86	77.5	119	176	86.2	22.1			Kase and Nakayama, 1974
KY 10440 (Met$^-$, AHV^R, AEC^R)	115	117	174	295	128	31.3			Kase and Nakayama, 1974
KY 10251 (Met$^-$, AHV^R, AEC^R)		111		107.3		95			Kase and Nakayama, 1974

Activity in the absence of added amino acids is taken as 100%.

All assays were performed in the absence of ammonium sulphate. The enzyme from ATCC 13059 was assayed at pH 7.0 AEC RN-13-6/1 at pH 7.5 and others at pH 8.0.

Cells of ATCC 13059 and AEC RN-13-6/1 were grown in medium C.

The Met$^-$, AHV^R) parent strain of KY 10440 responded to amino acids like KY 9159, whereas activation by L-threonine was lost in the (Met$^-$, AHV^R) parent strain of KY 10251.

AHV-L-α-amino-β-hydroxyvaleric acid.

derived from these two organisms, *M. glutamicus* AEC RN-13-6/1 and *B. flavum* FAI-30, are very similar to one another in their response to lysine, threonine and lysine plus threonine. While the property of stimulation by isoleucine and reversal of lysine inhibition by this amino acid was retained in the *M. glutamicus* mutant, this property was lost in the mutant of *B. flavum* (Shiio *et al.*, 1970). The patterns of desensitization to lysine and threonine inhibition are different in *M. glutamicus* AEC RN-13-6/1 and in the AEC^R mutants of *M. glutamicus* KY 9159 (table 6). This is probably due to the different methods employed in the selection of AEC^R mutants. The aspartokinase activities of various strains of *M. glutamicus* differ slightly in their response to the aspartate family of amino acids (table 6). Considering that the degree of inhibition or activation by feedback modifiers depends on the pH of the assay (Zaitseva and Chernysheva, 1975), the presence or absence of ammonium sulphate in the assay medium (Kase and Nakayama, 1974) and on growth medium composition, the significance of observed differences between strains should be evaluated carefully.

No genetic derepression of aspartokinase activity was detected in *M. glutamicus* AEC RN-13-6/1 (Lakshman, 1980). The specific activity of aspartokinase in crude extracts of *M. glutamicus* KY 10440 was 1.3 times higher than in its parent strain KY 9159 (Kase and Nakayama, 1974). On the other hand, the aspartokinase activity in crude extracts of the AEC^R mutant of *B. flavum* was 2.5 times higher than in the parent AEC-sensitive strain (Shiio *et al.*, 1970).

Isofunctional aspartokinases with differing repression and feedback inhibition patterns can be detected by observing feedback inhibition pattern in cells obtained from different growth media or during purification. They may also be detected by observing feedback inhibition patterns at different growth stages, provided the turnover rates of these enzymes differ. The data presented here, of the purification as well as the similarity in the pattern of feedback inhibition in different growth media and growth stages, failed to reveal more than one aspartokinase in *M. glutamicus* AEC RN-13-6/1. These experiments do not however conclusively rule out the possibility of the existence of multiple aspartokinases in this organism.

Dihydrodipicolinate synthetase in crude extracts of *M. glutamicus* has been reported to be insensitive to L-lysine (Nakayama *et al.*, 1966). Diaminopimelate decarboxylase of *M. glutamicus* was reported to be repressed slightly (10-20%) by 40 mM L-lysine and inhibited by L-lysine, competitively with respect to diaminopimelate (Drazic and Vitale, 1974). Repression of dihydrodipicolinate synthetase and the effect of amino acids other than L-lysine on the level of diaminopimelate decarboxylase have not been investigated. Data presented in this communication support and extend the earlier observation (table 4). Together with the observation that L-lysine, methionine, threonine and isoleucine have no repressive effect on aspartokinase (table 3), these data suggest that feedback inhibition of aspartokinase activity is the most important mechanism in end-product regulation of L-lysine biosynthesis in *M. glutamicus* AEC RN-13-6/1. Furthermore, the levels and regulatory properties of dihydrodipicolinate synthetase and diaminopimelate decarboxylase were the same in *M. glutamicus* AEC RN-13-6/1 and *M. glutamicus*

ATCC 13059 (Lakshman, 1980). Therefore, the major difference in regulation of lysine biosynthesis in these strains, which provides a plausible explanation for lysine over-synthesis by the former, was the feedback inhibition of aspartokinase.

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Nature of leukemia-associated antigenicity of dinitrophenylated human lymphocytes and lymphoblasts

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Abstract. Using dinitrophenylated human lymphocytes and phytohaemagglutinin-stimulated human lymphoblasts as antigens, antibodies were produced in rabbits. The immonological reactivities of the antisera so produced were tested against various types of leukemic cells after absorbing the sera with pooled normal leukocytes. Both the sera showed reactivity with all types of leukemic cells and no specific affinity for lymphoid leukemic cells was seen. This may suggest the presence of some common antigens on all types of leukemic cells or that dinitrophenylation brings about similar changes on all types of normal leukocytes.

Keywords. Dinitrophenylation; lymphocytes; lymphoblasts, leukoagglutination; immunoprecipitation; immunofluorescence; leukemia-associated antigens.

Introduction

It has been shown by Sahasrabudhe *et al.* (1971, 1972 a, b, 1974) and Prema *et al.* (1977, 1978) that the antisera raised against dinitrophenylated leukocytes showed a spectrum of activity against different types of leukemic cells. Absorption studies indicated that the sera raised against dinitrophenylated leukocytes contain probably a mixture of immunoglobulins, each having reactivity with specific leukemic cell types (Prema, 1972). It was suggested that this may be due to the use of a mixture of cells in the leukocyte preparation as antigen. In the present studies, an attempt was made to use as antigens dinitrophenylated human lymphocytes not contaminated with other cells to study the nature of antibodies and its specificity to various leukemic cell types. In addition, phytohaemagglutinin (PHA)-stimulated, human dinitrophenylated lymphoblasts were also used as antigens to produce an antiserum for possible use against acute human leukemia. For this purpose, lymphoblasts obtained by PHA stimulation were tagged with flurodinitrobenzene (FDNB) and these DNP-lymphoblasts were used for raising the antibodies in rabbits.

Abbreviations used: PHA, phytohaemagglutinin; FDNB, fluorodinitrobenzene; RBC, red blood cells; WBC, white blood cells; FITC, fluoroisothiocyanate.

Materials and methods

Materials

Phytohaemagglutinin (PHA) was purchased from Burroughs Wellcome, England; minimal essential medium (MEM) from SISCO Laboratories, Bombay; fluorodinitrobenzene (FDNB) from E. Merck, Darmstadt, West Germany; streptomycin and penicillin from Sarabhai Chemicals, Baroda; glass beads (diameter; 118-125 μ) from Haldynglass, Bombay and agar from Difco Laboratories, Detroit, Michigan, USA.

Preparation of lymphocytes

Lymphocytes were separated from the blood of normal healthy '*O*' group donors using the glass bead column method of Robinowitz (1963) with slight modifications. The lymphocytes contaminated with red blood cells (RBC) were eluted from the column and collected in a sterile bottle. The contaminating RBC present in the preparation were lysed and removed by ammonium chloride-Tris treatment as described by Boyle (1968). The lymphocyte preparations thus obtained were free of RBC contamination and were used either for direct immunization or for blast transformation with PHA.

Preparation of PHA-stimulated lymphoblasts

Lymphocytes obtained as described above were washed and suspended in minimal essential medium with 20% autologous serum and antibiotics (penicillin and streptomycin). The cell concentration was made to 2×10^6 cells/ml. PHA was then added to the suspension to a concentration of 20 μg/ml. These cells were incubated at 37°C for 72 h in an atmosphere containing 5% CO_2 and 95% air. After 72 h the cultures were tested for blast transformation. The PHA-transformed blast cells were then isolated and tagged with flurodinitrobenzene (FDNB) and injected into the rabbit immediately.

Dinitrophenylation of lymphocytes/lymphoblasts

Equal volumes of lymphocytes or lymphoblasts (10^7 cells/ml) and FDNB solution (corresponding to 10^{11} molecules, *i.e.* 31 pg of FDNB per ml in normal saline) were mixed and shaken for 10 to 15 min at room temperature. The pH was adjusted to 7.2 to 7.4 with $NaHCO_3$ (Eisen *et al.*, 1959). The unreacted FDNB and HF were removed by repeated washings with large volumes of saline. The tagged cells were re-suspended in sterile isotonic saline and used for immunizations.

Immunization of rabbits with DNP-lymphocytes or with DNP-lymphoblasts

Rabbits were immunized once every week for 12 weeks by subcutaneous injections of 2×10^7 cells of either DNP-lymphocytes or DNP-lymphoblasts. Ten days after the last immunization the rabbits were bled. The serum was heated at 56°C for 30 min to inactivate the complement. The non-specific antibodies were removed by repeated absorption of the serum with pooled white blood cell from a number of healthy subjects. These absorbed sera were used for testing the reactivity against different types of leukemic cells.

Isolation of human leukemic cells

Blood samples from leukemic patients were collected in heparinized tubes, and kept at 37°C for 30 min and the supernatant containing leukemic white blood cells (WBC) were separated. The cells were immediately washed five times with large volumes of normal saline to remove the anticoagulant. During the separation of WBC, the sample was centrifuged each time at 250 g for 5-10 min (Bert *et al.*, 1969). Minimum exposure of WBC to the anticoagulant (heparin) and low speed centrifugation was resorted to in order to minimize non-specific aggregation (Ling, 1968). Contaminating RBC, if any, were removed by hypotonic shock treatment.

Serological titration

The normal and leukemic WBC thus collected were suspended in normal saline to give a 5% cell suspension and this was used for serological titration. The titre value was evaluated by the double dilution technique. Serial dilutions of the antisera were made in normal saline (Amos, 1953; Killman, 1960; Kiesmeyer and Nielson, 1969). Saline (0.03 ml) containing 10^5 cells and variously diluted antisera (0.03 ml) were mixed in microprecipitin tubes (siliconized) and incubated at 37°C for 1 h (Killman, 1960). Following the incubation, the tubes were centrifuged at 250 *g* for 1 min to accelerate the agglutination which did not interfere with the titre values (Amos, 1953; Andre *et al.*, 1958). The degree of agglutinability was graded in accordance with the criteria provided by Pyne (1964). The maximum dilution of the serum which showed definite clumping (minimum +1) was taken as the titre of the antiserum. Positive and negative reactions were checked by microscopic examination (Pyne, 1964).

Fluorescent antibody techniques

The anti-DNP-lymphoblast serum absorbed with normal cells were conjugated with fluoroisothiocyanate (FITC) as described by Weir (1967). The FITC-conjugated serum was tested for its reactivity with leukemic cells. Single drops of cell suspension from normal human leukocytes or human leukemic leukocytes as the case may be, were mounted on microslides and one drop of FITC-conjugated immunoglobulin was added to each slide. These slides were kept in a moist chamber and incubated for 1 h at 37°C. After the incubation, the unreacted FITC-conjugated globulins were washed off completely with phosphate-buffered saline, pH 7.2, till the background showed no fluorescence. The cells were mounted with 10% glycerol and the coverslip was sealed with nail paint. The fluorescent characteristics of these cells were observed under a Nikon fluorescent microscope.

Measurement of electrophoretic mobility of the DNP-lymphocytes

The FDNB-tagged and untagged lymphocytes, PHA-stimulated lymphoblasts and leukemic cells were suspended in an electrophoretic medium containing 1.4 M NaCl and 0.14 M KCl, pH 7.2. The electrophoretic mobility of these cells was measured at 30°C using an electrophoresis apparatus (Rank Brothers, Bottisham, Cambridge, England). The technique of operating the instrument and the calculation were as described by Ambrose (1965). The mobility of the cells was expressed as µ/s/v/cm. Each value was obtained by timing the movements of atleast 50 cells with reversal of polarity after each reading. The alignment of the apparatus was checked by determining the mobility of washed human erythrocytes in normal saline.

Results and discussion

In table 1 the agglutination titre values of anti-DNP-lymphocyte serum against various types of leukemic cells are given. The titre values of the serum against chronic myeloid leukemic cells ranged from 64 to 4096. In the case of chronic lymphocytic leukemic cells, the titre ranged from 256 to 2048. The titre values of the serum against acute lymphocytic leukemia were between 512 and 4096 and against myeloid leukemic cells, the serum gave a titre between 1024 and 4096.

Table 1. Agglutination titre of the absorbed antiserum raised in rabbits with DNP-lymphocytes.

Cell types used	No. of cases studied	Agglutination titre
Chronic myeloid leukemia	15	64—4096
Chronic lympoid leukemia	4	256—2048
Acute lymphoid leukemia	8	512—4096
Acute myeloid leukemia	10	1024—4096
Normal cells	—	0—16

Agglutination titre (maximum dilution of the antiserum showing definite clumping of cells) was determined by the double dilution technique (Amos, 1953; Killman, 1960; Kiesmeyer and Nielson, 1959).

Since the serum was free of non-specific activity after absorption with normal WBC, no agglutination was seen with normal cells. Figure 1 shows the specificity of the anti-DNP-lymphocyte serum against the different types of leukemic cells as studied by the Ouchterloney gel diffusion technique. The serum did not form any precipitin line with normal cells, but formed a clear line with different types of leukemic cells.

Table 2 gives data on the agglutination titre values of the serum against various types of leukemic cells. The serum gave agglutination titre values against acute lymphoid leukemic cells ranging from 512 to 4096. In the case of acute myeloid leukemic cells, the serum gave a titre of 256-4096. The cells from chronic myeloid leukemic patients with blast crisis reacted with a titre of 1024-2048. On the other hand, in the case of chronic myeloid leukemic cells the serum gave a titre ranging from 128 to 8192. The serum showed the minimum titre with normal cells.

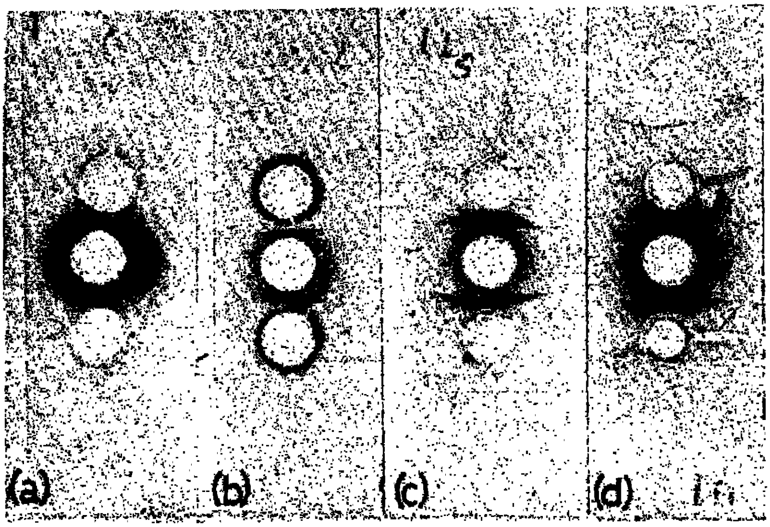

Figure 1. Agar gel diffusion studies showing cross reactivity of the anti-DNP lymphocytic serum with (a) normal cells (b) chronic myeloid leukemia cells (c) chronic lymphocytic leukemia cells and (d) acute lymphoid leukemia cells.

Leukemic and normal WBCs were lysed separately in distilled water and centrifuged at 1000-1500 rpm for 15 min. The sedimented crude membraneous material was taken in saline and a few drops of 0.1 N NaOH were added to dissolve the sediment. This solubilized membraneous material was used to study the precipitin reaction of the antisera.

Table 2. Agglutination titres of absorbed antiserum raised in rabbits with DNP-lympoblasts.

Cell types used	No. of cases studied	Agglutination titre
Acute lymphoid leukemia	10	512—4096
Acute myeloid leukemia	9	256—4096
Cells from chronic myeloid leukemia blast crisis	5	1024-2048
Chronic myeloid leukemia	15	128—8192
Normal leukocytes	—	16—32

Figure 2 shows the specific precipitin reaction of the anti-DNP-lymphoblast serum with different types of leukemic cells. Leukemic cells gave clear precipitin lines while normal cells failed to do so. Further, figure 3 shows the reactivity of the anti-DNP lymphoblast serum conjugated with FITC, against leukemic and normal cells. FITC-conjugated anti-serum did not show appreciable fluorescence with normal cells; the same showed bright fluorescence with leukemic cells.

Table 3 shows the electrophoretic mobilities of DNP-tagged and untagged lymphocytes and PHA-stimulated lymphoblasts and of leukemic cells. It is clear from the data that the net surface charge of the dinitrophenylated cells were more

negative than their normal counterparts and also were comparable to t
leukemic cells.

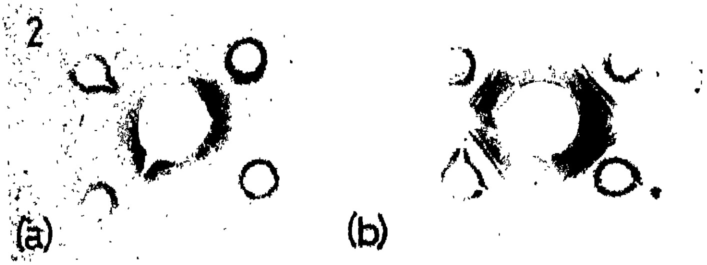

Figure 2. Ouchterloney's agar gel diffusion studies showing cross-reactivity of anti-D
lymphoblast serum with (a) normal leukocytes and (b) leukemic leukocytes.

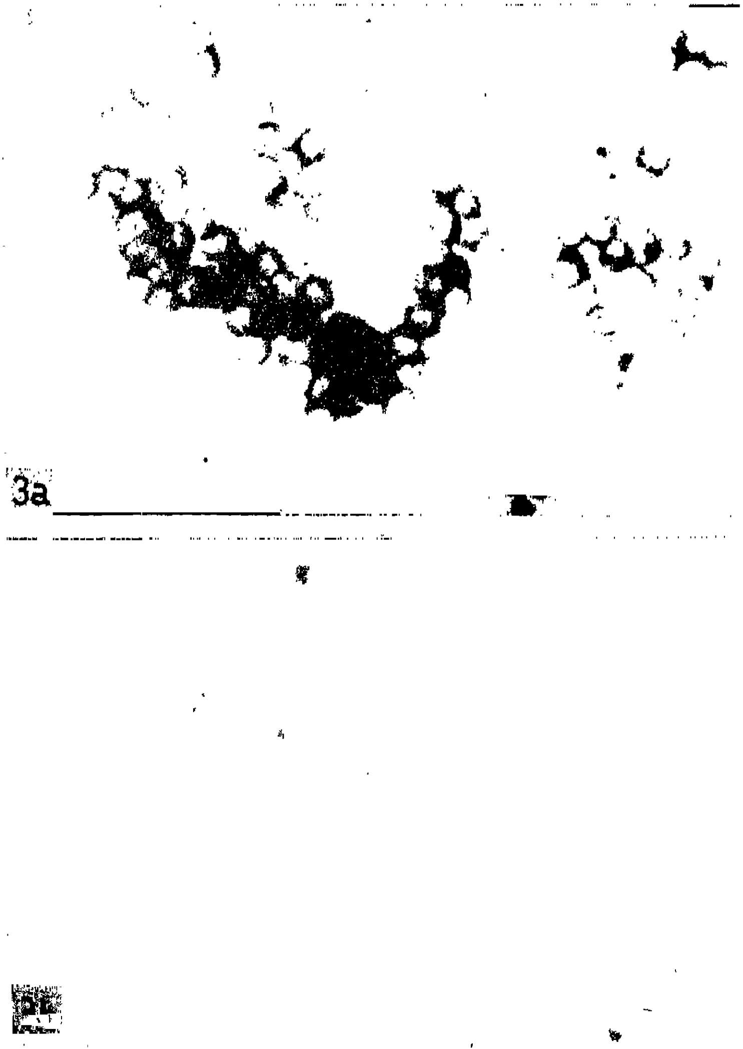

Figure 3. Immunofluorescence studies with FITC-conjugated anti-DNP-lymphobl
(a) leukemic cells showing bright fluorescence and (b) normal cells showing faint flou

Table 3. Electrophoretic mobilities of FDNB tagged and untagged normal human lymphocytes and PHA-stimulated lymphoblasts.

Type of cells	Electrophoretic mobility (μ/s/v/cm) (mean of 10 values)
Normal lymphocytes	−1.258 ± 0.085
DNP-lymphocytes	−1.714 ±0.065
PHA-stimulated lymphoblasts	−0.980 ±0.015
PHA-stimulated DNP-lymphoblasts	−1.370 ±0.035
Leukemic leukocytes	−1.878 ±0.065

The results described in this paper show that even when the purified lymphocytes were used as antigens, the antiserum reacted with all types of leukemic cells. The exact mechanism of exposure of leukemia associated antigens of leukocytes following dinitrophenylation is not known. Karande and Sahasrabudhe (1978) have shown that anti-leukemic activity of the anti-DNP-leukocyte serum is not due to anti-DNP antibodies. Dinitrophenylation of cell surface amino groups by FDNB probably causes a transformation of hydrophilic ionic groups. Since the presence of hydrophobic groups on the cell surface exposed to aqueous environment is thermodynamically unstable (Singer, 1971), a tendency for the newly formed hydrophobic groups to sink deeper into the inner hydrophobic environment is expected. This might have caused conformational changes, with consequent exposure of neo-antigenic sites on the cell surfaces. The present study emphasizes that the immune response elicited is more general than specific. This may also suggest that different leukemic cells probably share common antigens.

Metzar *et al.* (1972, 1975, 1977), Mohankumar *et al.* (1974, 1975) and Greaves (1975) have reported the production of antileukemic antisera in rabbits which showed cross-reactivity with different morphological classes of leukemic cells. Metzar and Mohankumar (1978) also showed that when antisera were produced in non-human primates, the antisera could distinguish the different histological types of leukemic cells. The capacity of different antigens to distinguish the different histological types of cells depends on the relative nearness or similarity of the species contributing the antigenic cells and the species in which the immunological response is raised. In the present study, rabbits were used for eliciting immune responses. This may be the reason for antisera raised in rabbits not being able to differentiate between different histological types of human leukemic cells.

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Animal model for testing human Ascaris allergens

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Abstract. Guinea pigs immunized through the nasal route by an antigenic preparation of human *Ascaris lumbricoides* produced predominantly homocytotropic antibodies. The sensitization of the homologous skin required a latency period. Hypersensitivity reaction was triggered within 30 min. of the antigen challenge. The antibodies were sensitive to heat and β-mercaptoethanol treatments and appeared to be similar to the IgE tvpe of immunoglobulins of man and rabbit. The antigenic preparation elicited an immediate type of skin hypersensitivity reaction also in human subjects harbouring the Ascaris parasite. The guinea pig model appears suitable for testing the activity of Ascaris allergens.

Keywords. *Ascaris lumbricoides*; homocytotropic antibodies; IgE immunoglobulins; guinea pig.

Introduction

Incidence of hypersensitivity to Ascaris antigens has been observed among the laboratory workers infected by the worm (Sadun, 1972). During the purification of trypsin (Mukerji *et al.*, 1976) and chymotrypsin (Mukerji *et al.*, 1977) inhibitors from *Ascaris lubricoides* var. *hominis* in this laboratory, some of the workers showed immediate hypersensitivity reaction to the trichloracetic acid-soluble preparation of the crude inhibitors. Attempts were made to purify the allergenic factor and an animal model was developed to test the allergen. The details of this model are described in this paper.

Materials and methods

Collection of worms and extraction of allergens

Ascaris lumbricoides var. *hominis* were collected from both in-patients and out-patients of King George's Medical College, Lucknow, and were processed according to the method described earlier (Mukerji *et al.*, 1976). The trichloroacetic acid-soluble material and the first peak, obtained by gel filtration through Sephadex-G-75 (Mukerji *et al.*, 1976) were used as the source of allergens for standardization of the animal model. Trichloroacetic acid was removed by extensive dialysis against distilled water (500 ml changes every 4 h) at 4°C.

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Immunization schedule

Earlier studies (Pathak, 1977) from this laboratory reported that the guinea pig was a suitable model for testing the activity of the allergens isolated from the pollens of *Chenopodium album*. The same animal species was used in the present study. Male guinea pigs (250-300 g) were administered 0.1 ml of allergen solution (2 mg/ml in 150 mM NaCl) in each of the nostrils by a dropper. The dose was repeated every 4th day till 6 such doses were given. The animals were bled one week after the administration of the last dose, serum was collected, inactivated to destroy complement and stored at −10°C. A group of animals was also immunized by the same schedule using a subcutaneous route.

Passive skin tests

Normal guinea pigs were sensitized by intradermal administration of 0.1 ml of the immunized guinea pig serum on preshaven skin. After 24 h the animals were challenged with the antigen at the sensitized site and the area of wheal formed was marked after 30 min unless otherwise mentioned, and the area measured. The area of wheal formed in the unsensitized guinea pig skin (diameter 10 mm) was subtracted from the experimental reading.

Skin tests in Ascaris positive subjects

Skin test was performed on human subjects, who were infected with Ascaris as determined by stool examination, by intradermal injection of 0.05 ml allergen solution (10 µg/ml protein) in the forearm and the area of the wheal was measured.

Heat stability of antibodies

Sera of the immunized animals were heated for 2 h at 56°C followed by immediate cooling in crushed ice.

β-*Mercaptoethanol treatment*

The antibodies were treated with β-mercaptoethanol essentially according to the method of Dobson *et al.* (1971). Serial dilutions of the immune serum were incubated with equal vol. of 200 mM β-mercaptoethanol at 37°C for 1 h followed by dialysis against 100 vol of 200 mM iodoacetamide for 24 h. The material was further dialyzed against 3 changes of (100 volumes) phosphate buffer saline (100 mM phosphate buffer, pH 7.2; 150 mM NaCl).

Precipitating and agglutinating antibodies

Detection of precipitating and agglutinating antibodies was made by immunodiffusion in agar and by the indirect haemagglutination method respectively as described by Kabat and Mayer (1961).

Results

Antibody response in guinea pigs immunized by the nasal and subcutaneous routes

Guinea pigs immunized by the nasal route exhibited a strong skin reaction within 30 min of the antigen challenge. However, precipitating and agglutinating antibodies were not detected in the sera of these animals. Animals immunized by the subcutaneous route did not display significant hypersensitivity reactions following the antigen challenge. The presence of precipitating antibodies also could not b seen in the sera of these animals. However, by indirect haemagglutination a weak titre of antibodies (½) could be seen.

Characterization of the cytotropic antibodies

Homocytotropic nature

Table 1 shows the data on skin test in guinea pigs passively sensitized with the serum from immunized animals. Strong reaction was observed upto 1/16 dilution of serum. The response was reduced at subsequent dilution but was still detectable at 1/128 dilution.

Table 1. Antibody titre of guinea pig serum immunized by the nasal route

Antiserum dilution	Area of wheal (mm^2)[a]
1/4	165
1/8	158
1/16	134
1/32	113
1/64	88
1/128	78
1/256	12

[a] Antisera from 3 guinea pigs were pooled. Each value is the mean of skin tests in two different animals. The area of skin raised due to injection of 0.15 M NaCl has been substracted from the experimental readings.

Time course of optimal binding of antibodies

Guinea pigs sensitized with a constant volume of the antiserum were challenged at different periods after the passive transfer. Significant skin reaction was not visible before 6 h of the antibody transfer (table 2). The response was better after 24 h and was maintained till the end of the observation period (72 h).

Table 2. Sensitization of homologous skin as a function of time

Time after transfer of antiserum (h)	Area of Wheal (mm^2)[a]
3	28
6	63
24	132
48	132
72	103

Each value is the mean of two skin tests in two different animals.

Onset and duration of hypersensitivity reaction

Table 3 presents the data on skin reactions measured at different periods after the allergen challenge in the passively sensitized animals. Skin reaction of a fairly large magnitude appeared within 30 min of the challenge with a slight increase in the value in the subsequent period up to 3 h. A gradual decrease in the wheal area was noticed by 12 h and the reaction subsided by 24-48 h.

Table 3. Onset and duration of hypersensitivity reaction

Time after antigen challenge (h)	Area of wheal (mm²)[a]
0.5	103
1.0	135
2.0	154
3.0	154
12.0	113
24.0	32
48.0	0

[a] Each value is the mean of two skin tests in two different animals.

Sensitivity of the antibodies to heat and β-mercaptoethanol

Significant reduction in the skin reaction was observed after heating the antiserum at 56°C for 2 h or its treatment with β-mercaptoethanol (table 4). Later treatment resulted in complete inactivation at 1/64 dilution.

Table 4. Sensitivity of antibodies to heat and β-mercaptoethanol treatments

	Area of wheal (mm²)		
Antiserum dilution	Untreated	Heated	β-mercaptoethanol treated
1/32	113	38	28
1/64	86	19	0
1/128	78	0	0

Skin response in Ascaris positive subjects

With a view to ascertain if the antigenic fraction of human Ascaris that elicited immediate hypersensitivity response in the sensitized guinea pigs also produced comparable response in human subjects, the skin test was performed in individuals who were positive to Ascaris by stool examination. All the cases examined gave a skin reaction of strong or medium grade upon challenge with 1 µg antigen protein (table 5). Ascaris negative patients, however did not show any response.

Table 5. Skin test with human Ascaris antigen(s) in Ascaris positive and Ascaris negative subjects.

Subjects	Area of wheal (mm^2)[a]				
	1	2	3	4	5
Ascaris positive	98	190	167	154	185
Ascaris negative	2	0	0	30	12

[a] Each value is mean of two determinations on the same patient.

Discussion

The immunization schedule followed in the present study succeeded in eliciting a failry high titre of homocytotropic antibodies in guinea pigs immunized by the nasal route. The precipitating antibodies of IgG class which might suppress the synthesis of reagenic antibodies and interfere with the hypersensitivity reaction by interacting with the allergen, thereby preventing it from reacting with the sensitized mast cells, were not formed by this schedule.

The observations on the requirement of a latency period for sensitization of homologous skin, persistence of sensitization for a few days, appearance of the skin reaction within 30 min of the antigen challenge and a substantial recovery by 12 h provide confirmatory evidence in favour of the homocytotropic nature of the antibodies that give rise to the immediate type of hypersensitivity reaction (Dobson *et al.*, 1971; Hussain *et al.*, 1973). Guinea pig antibodies similar to the IgE type of human antibodies are characterized by their sensitivity to heat and β-mercaptoethanol (Dobson *et al.*, 1971). The data of the present study on heat and β-mercaptoethanol treatment suggest a fairly good concentration of IgE type of antibodies in the sera of immunized guinea pigs. A small amount of homo-cytotropic antibodies belonging to $7Sr_2$ type (Hussain *et al.*, 1973) were also present as the reaction continued to increase till 3 h.

The data on skin reaction in Ascaris-positive human subjects show that the antigen fraction which elicits a hypersensitivity reaction in guinea pigs also triggers the skin response in man. Guinea pigs sensitized with the human Ascaris antigen, therefore, are a satisfactory model for testing human Ascaris allergens.

Experimental hypersensitivity to *A. suum* antigens has been produced in the guinea pig, by injecting embryonated eggs (Strejan and Campbell., 1967 a, b) or repeated administration of Ascaris extract by intramuscular or subcutaneous routes (Strejan and Campbell, 1970, 1971). Immunization under these conditions requires longer time and a larger quantity of the antigen. Moreover, in addition to the skin binding antibodies the sera of the immunized animals also contain an appreciable titre of precipitating antibodies which may interfere with the skin reaction.

Human Ascaris allergen appears to be a glycoprotein. Studies on its characte zation will be reported elsewhere.

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Effect of human chorionic gonadotropin on serum levels of progesterone and estrogens in the pregnant bonnet monkey *(Macaca radiata)*

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Abstract. Administration of human chorionic gonadotropin to pregnant bonnet monk (*Macaca radiata*) at 55-60 days and 130-140 days of pregnancy resulted in a significant incre in serum progesterone levels. This effect could be observed even in lutectomized monke However, no significant change in the serum estrogen level was noticed. These results sugg that although no chorionic gonadotropin is detectable in the serum after 35 days of pregnan the foetoplacental steroidogenic system is still responsive to exogenous gonadotro stimulation.

Keywords. Placenta; chorionic gonadotropin; steroidogenesis.

Introduction

It has been suggested by Neill and Knobil (1972) that in the rhesus monkey, the lo levels of chorionic gonadotropin released at the time of and subsequent t implantation is responsible for the transient restoration of the progesterone-secretory function of the corpus luteum of the cycle during very early stages of pregnancy. The decline in the progesterone levels during early pregnancy (by 23-2 days) is suggested to be due to the refractoriness of the corpus luteum to chorioni gonadotropin (Neill and Knobil, 1972). The chorionic gonadotropin level in th serum reaches a peak value by about the 23rd day of pregnancy and decrease rapidly thereafter to an undetectable level by the 35th day (Hodgen *et al.*, 1974; Atkinson *et al.*, 1975). The placental level of chorionic gonadotropin also becomes undetectable, by the 40th day (Hodgen *et al.*, 1975). In spite of the lo serum progesterone level (Neill *et al.*, 1969; Bosu *et al.*, 1973) the levels in the utero-ovarian and uterine venous system are high (Walsh *et al.*, 1974), suggesting that the serum progesterone level may not be a good indicator of progesterone synthesis in specific tissues during pregnancy. Pregnancy in the bonnet monkey can be terminated upto the 25th day by administration of an antiserum raised to ovine LH or

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Abbreviation used: hCG, Human chorionic gonadotropin.

LH β in the monkey (Prahalada *et al.*, 1975; Moudgal *et al.*, 1978). It is also shown that ovareactomy after the 21st day of pregnancy has no effect on the course of pregnancy (Tullner and Hertz, 1966). These results emphasize that the ovarian function in the maintenance of pregnancy is limited to the first 21 days and that the placenta may be taking over some of the functions of the ovary, especially steroidogenesis. In this paper, we report results which suggest that the placenta in the monkey is responsive to exogenous administration of human chorionic gonadotropin (hCG), by producing progesterone at a time even when endogenous chorionic gonadotropin is not detectable.

Materials and methods

Chemicals and methods

Highly purified hCG (9000-10000 I.U./mg) was obtained as a gift from the National Institute of Health, Bethesda, Maryland, USA. Radioimmunoassays of serum progesterone and estrogen were done according to the procedures validated in the laboratory (Prahalada *et al.*, 1975).

Animals

Adult cycling female bonnet monkeys weighing 4-6 kg each exhibiting three successive regular cycles were employed in the study. The animals were housed in individual cages in rooms with controlled lighting regimen of 12 h light per day The menstrual cycles of these monkeys ranged from 25-28 days and the first day o menses was considered as day one of the cycle. Females were exposed to male monkeys of proven fertility for four days (day 10 through 13) of the cycle. Detail of the breeding programme and the method of monitoring pregnancy have been described (Madhwa Raj *et al.*, 1974; Moudgal *et al.*, 1974; Prahalada *et al.*, 1975) The animals were fed with pelleted monkey feed from Hindustan Lever Limited Bombay and water was provided four times in a day. Besides, the diet wa supplemented with fresh carrots and bananas every day.

Lutectomy

Pregnant monkeys (monkey no. 79, 55-60 days and monkey no. 96, 135-140 days were subjected to lutectomy under intraval sodium (May and Baker, Bombay, 2 mg/kg body weight) anaesthesia. The corpus luteum was excised completely b making a half-inch midventral incision. The animals were allowed a minimum of days' rest before subjecting them to further experimentation.

Injection and bleeding

Pregnant monkeys were injected intravenously with 1 mg of hCG in sterile saline 9 A.M. Blood (5 ml) was collected at specified intervals from the femoral vei Serum was separated and stored at −20°C. All monkeys were injected Unifer with B_{12} (Glaxo Laboratories, Bombay) during the experimental period compensate for the loss of blood. Two pregnant monkeys injected 1 ml of 0.9 sodium chloride served as controls.

Results

Effect of hCG during pregnancy

It can be seen from figure 1 that both the early pregnant monkeys (No. 88, 508 responded to hCG injection by a marked increase (5-7 fold) in serum progesteron reaching a peak value at 6 h. Similarly, the late pregnant monkey (No. 50) als responded to exogenous hCG by an increased serum progesterone level. It ca also be seen that serum progesterone levels increased significantly (2 to 3-fold) a early as 2 h.

Effect of lutectomy

After ensuring that the pregnant monkeys (No. 79 and 96) responded to exogenou hCG, in an identical manner compared to those shown in figure 1, the corpora lutea were excised, hCG administered and the progesterone levels monitored at the time intervals indicated in figure 2. An increase in progesterone levels similar to that observed in the non-lutectomized monkey (figure 1) was observed with these lutectomized monkeys. The peak occurred at 12 h compared to 6 h in the normal monkeys.

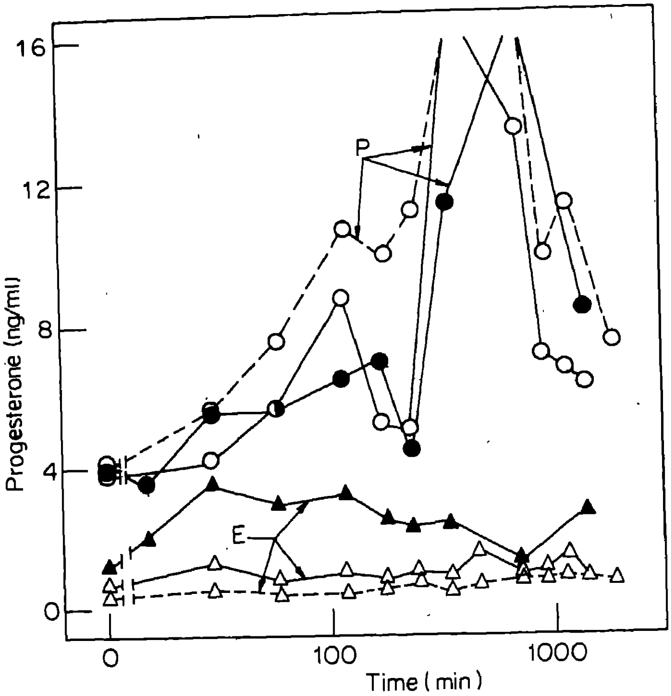

Figure 1. Effect of injection of hcG on serum levels of progesterone and estrogens during early pregnancy.
Early pregnancy (Monkey No. 88,508): Progesterone (O), Estrogens (Δ). Late pregnancy (Monkey No. 50: Progesterone (●), Estrogens (▲).

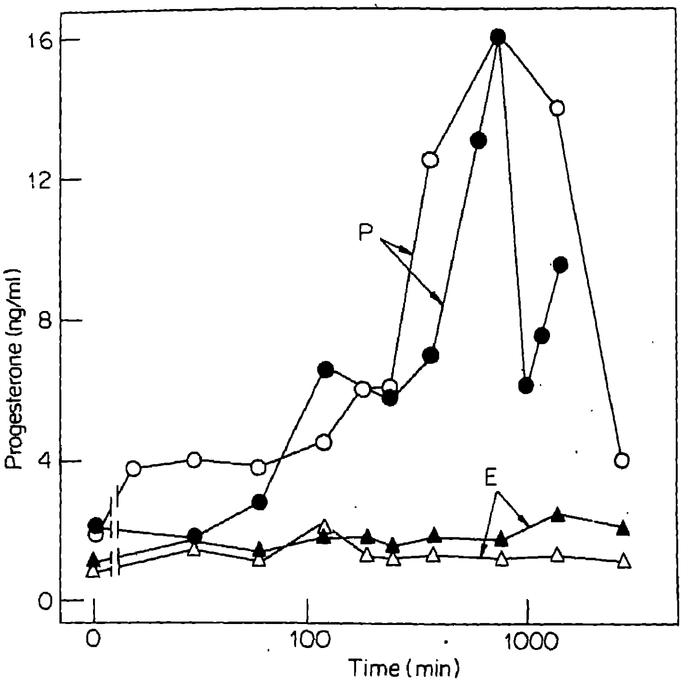

Figure 2. Effect of hcG on serum levels of progesterone and estrogens in leutectomized monkey.
Early pregnancy (Monkey No. 79): Progesterone (O), Estrogen (Δ). Late pregnancy (Monkey No. 96): Progesterone (●), Estrogen (▲).

One possible explanation for the increased progesterone levels could be the reported diurnal variation in progesterone levels (Bosu *et al.*, 1973). To examine this possibility a control study was carried out by injecting saline instead of hCG and monitoring the progesterone levels (figure 3). It can be seen that the diurnal flutuation is only from 2 to 5 ng compared with 4 to 25 ng in the hCG-treated animals.

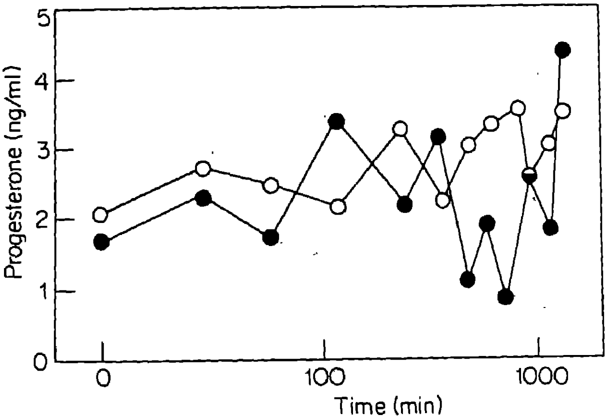

Figure 3. Effect of injection of saline on serum levels of progesterone.
Early pregnancy (Monkey No. 79), (O)
Late pregnancy (Monkey No. 96), (●).

Discussion

It is well known that the corpus luteum is transiently stimulated by chorionic gonadotropin in the monkey and this stimulation is responsible for the increased serum progesterone observed during early pregnancy,. During the late stages, this production of progesterone is taken over by the foetoplacental unit. The large increase in the progesterone level even in the lutectomized pregnant monkeys in response to hCG administration clearly demonstrates that the foetoplacental unit is responsive to exogenous gonadotropin. At this stage of pregnancy the chorionic gonadotropin is not detectable in the serum (Hodgen *et al.*, 1974; Atkinson *et al.*, 1975) and in placenta (Hodgen *et al.*, 1975) and it is not clear how the relatively high progesterone levels are maintained. Though the observation of Hobson (1972 a,b) that chorionic gonadotropin can be extracted from the placenta of 7-25 weeks in rhesus monkey has not been confirmed, it is possible that the placenta may be producing a very small quantity of chorionic gonadotropin not detectable by present methods. Also, such low quantities may be locally sequestered and stimulate steroidogenesis. The results of the present study show that the foetoplacental steroidogenic system of late pregnancy is responsive to exogenous chorionic gonadotropin and lend some support to such a suggestion.

Studies of Ainsworth *et al.* (1969) suggest that the placenta of the rhesus monkey possesses the enzymatic system required for the conversion of androgen precursors to oestrons and oestradiol as well as pregnenolone to progesterone and other progestogens *in vitro*. It is known that the biosynthesis of the steroids in the placenta depends on the precursors brought to it from the foetal and maternal compartments and the importance of foeto-placental pathways of steroid biosynthesis in the monkey has not been fully assessed (Bosu *et al.*, 1973). It is suggested that in the monkey, the foetus may be involved in steroid biosynthesis during pregnancy, and the maternal and foetal adrenals may also contribute to steroidogenesis during pregnancy (Bosu *et al.*, 1973). In fact it has been demonstrated in the rhesus monkey that during late pregnancy the foetal pituitary is responsive to exogenous gonadotropin releasing hormone and the gonad responds to hCG with increased testosterone production (Huhtaniemi *et al.*, 1977). Although the results obtained in the present study do not permit us to specify the site of action of hCG, they do suggest that the steroidogenic system of the foeto-placental unit in the monkey is responsive to exogenous hCG at a time when the influence of endogenous chorionic gonadotropin is completely absent.

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Purification and properties of diaminopimelate decarboxylase of *Micrococcus glutamicus*

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Abstract. Diaminopimelate decarboxylase (EC 4.1.1.20) of *Micrococcus glutamicus* ATCC 13059 was purified to homogeneity. The enzyme had an apparent molecular weight of 191,000 as determined by gel filtration on Sephadex G-200. At protein concentrations of 20 and 10 μg per ml and in the absence of pyridoxal-5'-phosphate, it dissociated into a species of molecular weight 94,000. The polypeptide chain molecular weight as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis was 100,000. The K_m for *meso* diaminopimelate was 0.5 mM and that for pyridoxal-5'-phosphate was 0.6 μM. Sulphydryl groups and pyridoxal-5'-phosphate were essential for activity and stability. The enzyme was inhibited significantly by L-lysine and DL-aspartic β-semialdehyde.

Keywords. *Micrococcus glutamicus;* diaminopimelate decarboxylase; pyridoxal phosphate; *meso*-diaminopimelate.

Introduction

Diaminopimelate decarboxylase (*meso*-1,7-diaminopimelate carboxylase EC 4.1.1.20, abbreviated as diaminopimelate decarboxylase), the terminal enzyme of the lysine biosynthetic pathway in bacteria and in higher plants, has not been purified before from any source to homogeneity. The enzyme from *Escherichia coli* was purified 200-fold (White and Kelly, 1965). This enzyme preparation sedimented as a single peak but gave four bands on gel electrophoresis. The major band, representing 70% of the protein was assumed to be the decarboxylase. The enzymes has been purified 150 to 170-fold from *Lactobacillus arabinosus* (Norton and Chen, 1970) and from wheat germ (Mazelis and Creveling, 1978). The homogeneity of these preparations was not checked. The enzyme from *Micrococcus glutamicus* ATCC 13286 (a homoserine auxotroph) has been purified 350-fold (Vitale *et al.*, 1977). This preparation contained three other proteins as evidenced by its electrophoretic pattern on polyacrylamide gels.

This paper describes the purification and properties of the diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059, a wild-type strain. Several differences were observed between this enzyme and that reported from *M. glutamicus* ATCC 13286.

Abbreviations used: SDS, sodium dodecyl sulphate; buffer PM, 0.1M potassium phosphate buffer, pH 7.5, containing 20 mM 2-mercaptoethanol; pyridoxal—P, pyridoxal-5' phosphate.

Materials and methods

Chemicals. Most of the fine chemicals used were the products of Sigma Chemical Co. (St. Louis, Missouri, USA). (DL+*meso*)-2,6-diamino $[1,7-{}^{14}C]$ pimelic acid was obtained from the Radiochemical Centre (Amersham, UK). All other chemicals were of analytical reagent grade. Commercial diamino $[1,7-{}^{14}C]$ pimelic acid was diluted to the desired specific activity with *meso*-diaminopimelate. The DD- and LL-isomers together constituted less than 1% of the resulting diaminopimelate samples. *Meso*-diaminopimelate was isolated from commercial diaminopimelate by fractional crystallization by the method of Work (1963). It was chromatographically pure and was completely decarboxylated to lysine by purified diaminopimelate decarboxylase. LL-diaminopimelate was isolated from the mother liquor obtained after the first crop of *meso*-diaminopimelate crystals were removed, by preparative paper chromatography (Work, 1962a). DL-aspartic-β-semialdehyde was prepared by ozonolysis of DL-C-allylglycine by the method o Black and Wright (1955). Diaminopimelate-sepharose was prepared by coupling synthetic diaminopimelate containing 36% of the *meso*-isomer, to AH-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) using 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide according to instructions provided by the manufacturer It should contain at least 0.38 μmol of diaminopimelate per ml gel for satisfactory resolution.

Bacteria and growth

M. glutamicus ATCC 13059 was maintained on slants of peptone-yeast extrac agar. Cells were grown in a medium containing glucose, 15%; NH_4Cl, 1% $MgSO_4.7H_2O$, 0.02%; urea, 0.25% K_2HPO_4, 0.01% (all constituents, w/v) and clarified corn steep liquor, 20% (v/v). Urea, K_2HPO_4 and corn steep liquor were autoclaved separately. pH was adjusted to 7.0 with ammonia just before inoculation. Corn steep liquor was clarified as follows: it was diluted with an equa weight of deionized water, neutralized to pH 7.0 with 10N NaOH, stirred continuously for 30 min and centrifuged at 3000 *g* for 10 min. The pellet wa washed thrice with deionized water so that the volume of the pooled supernatan and washings was four times the weight of the corn steep liquor taken. The clarified corn steep liquor was autoclaved at 1.1 kg cm^{-2} for 20 min. A second precipitat formed. The supernatant was either collected by centrifugation under asepti conditions or by decantation and used in the medium.

Cells were grown to late exponential phase (16 h) in 1 litre Erlenmeyer flasks on rotary shaker at 30°C. They were harvested in cold, washed three times with larg volumes of 0.03 M potassium phosphate buffer, pH 7.5, containing 0.02 M 2 mercaptoethanol and stored at −20°C until the preparation of cell-free extract.

Enzyme assay. Diaminopimelate decarboxylase was routinely assayed b measuring the rate of diaminopimelate disappearance as described by Whit (1971). The assay system of final volume 1 ml contained per ml, Tris.HCl buffer pH 7.5, 100 μmol; *meso*-diaminopimelate, 5 μmol; pyridoxal phosphate, 10 nmo and enzyme. A unit of activity is the amount of enzyme that catalyzes the di appearance of 1 μmol diaminopimelate per min at 37°C and specific activity the number of units per mg protein. The enzyme was also assayed by meas $^{14}CO_2$ released from diamino 1[1,7−^{14}C] pimelate (Kobayashi, 1963). The system was the same as that described above, except that radioactive substrat used. The reaction was terminated after 10 min by tipping in 0.2 ml of 6 N H_2 $^{14}CO_2$ was trapped in 10-20 µl hyamine hydroxide and counted in a Beckman m LS-100 liquid scintillation spectrometer.

Substrate specificity. Carbon dioxide released from compounds teste substrates for diaminopimelate decarboxylase was measured manometri (Work, 1962). The main compartment of the Warburg flask contained in 0.9 imidazole buffer, pH 7.0, 100 µmol; pyridoxal phosphate, 10 nmol and enzyme µg. One side arm contained the test compound in 0.1 ml; the other contained citric acid, 0.1 ml. Total gas evolved in 1 h at 37°C was measured by tippin citric acid. The values were expressed as those above a blank without substrat

Determination of molecular weight. The molecular weight of the enzyme determined by gel filtration at 4°C on a column (1.5×137 cm) of Sephadex G- by the method of Whitaker (1963). Bovine thyroglobulin (670,000), bovine se albumin (67,000; 134,000) and ribonuclease from bovine pancreas (13,600) w used as markers.

Electrophoresis. Disc gel electrophoresis was carried out using formulat described by Davis (1964) and Gabriel (1971). Sodium dodecyl sulphate polyac mide gel electrophoresis was carried out by the method of Weber *et al.* (19 Samples were heated in a boiling water bath in 0.01 M sodium phosphate buffer, 7.0, in the presence of 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol f min; cooled and dialyzed against running buffer before electrophoresis. Isoelect focussing was carried out in 5% polyacrylamide gels containing 0.8% Ampholi (Leaback and Rutter, 1968) in an LKB multiphor unit [LKB Produketer, Bromn Sweden].

Gels were stained by the method of Fairbanks *et al.* (1971). They were scann in a Joyce Loebl Chromoscan using a 620 nm filter. Enzyme was located in g after electrophoresis by slicing them into pieces of 5.0 mm length, homogenizi with 0.5 ml of 0.1 M buffer PM, pH 7.5, left overnight at 5°C and assayi qualitatively and colorimetrically, the activity in an aliquot of the supernatant.

Miscellaneous. To remove bound pyridoxal phosphate, hydroxylamine w added to the enzyme solution to 0.5 mM. The enzyme (11 µg/ml) was th dialyzed against 100 volumes of 0.1M potassium phosphate buffer pH 7 containing 0.02M 2-mercaptoethanol and 0.5 mM hydroxylamine for 30-40 mi This was followed by dialysis against the same buffer without hydroxylamine (volumes) for at least 3 h with rapid stirring and change of buffer every 30 min.

Results

Enzyme purification

All purification steps were carried out at 4-8°C. Thawed cells were ruptured b grinding with three times their weight of alumina (Type 305, Sigma) and extracte with buffer PM (5 ml/g wet cells) with grinding (Gunsalus, 1955). The extract wa centrifuged at 16,000 *g* for 10 min and the residue re-extracted with buffer PM (3 ml/g wet cells). The two extracts were pooled and centrifuged once more at 16,000 *g* for 10 min to remove alumina that might have decanted over.

A 25% solution of streptomycin sulphate in buffer PM was added dropwise to the cell-free extract until a final streptomycin concentration of 2% (w/v) was reached. The solution was stirred well and kept overnight. Precipitated matter was removed by centrifugation at 16,000 *g* for 10 min.

The streptomycin sulphate supernatant was fractionated with ammonium sulphate at pH 7.5 (Green and Hughes, 1955). Proteins precipitating within 30 min between 45% and 60% saturation with ammonium sulphate were collected by centrifugation at 25,000 *g* for 20 min. They were taken up in buffer PM and dialysed exhaustively against the same buffer. The dialyzed enzyme solution was chromatographed on a column of DEAE-Sephadex (figure 1). Fractions containing the enzyme activity were pooled and concentrated by ultrafiltration through a PM 10 membrane (Amicon BV, Oosterhout NB, Holland).

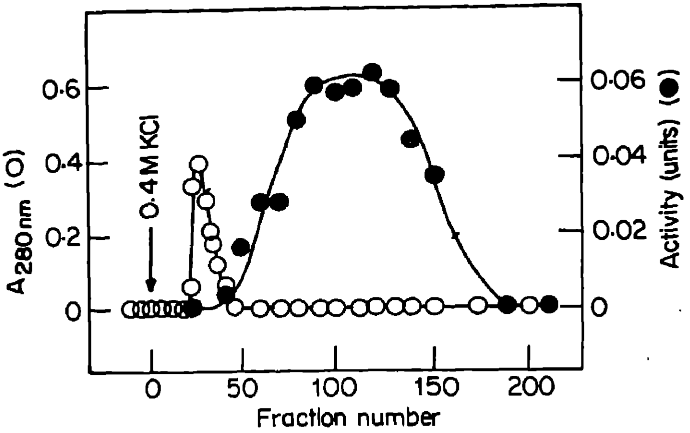

Figure 1. Chromatography of the 45-60% saturation ammonium sulphate fraction on DEAE-Sephadex. An aliquot containing 1,549 mg protein and 103 units of enzyme activity was loaded onto a column (3.3×20cm) of DEAE-Sephadex A-50 equilibrated with buffer PM. The column was washed with buffer PM and then buffer PM containing 0.3 M KCl until the absorbance of the effluent at 280 nm was less than 0.05. The column was subsequently developed with buffer PM containing 0.4 M KCl. The flow rate was 71 ml per hour. Fractions 50-170 were pooled. Protein, O, activity, ●.

The DEAE-Sephadex eluate was adjusted to 0.2 M KCl by the addition of buffer PM and chromatographed on a column of diaminopimelate-Sepharose (figure 2A). Fractions containing enzymatic activity were pooled, dialyzed against buffer PM and rechromatographed on diaminopimelate Sepharose (figure 2B). Considerable loss of activity occurred on rechromatography. Further loss of activity could be prevented by the addition of 5 µM pyridoxal-*P* to the enzyme preparation. The eluate of the second diaminopimelate-Sepharose column was dialyzed against 0.01 M potassium phosphate buffer containing 7.5 mM 2-mercaptoethanol and 5 µM pyridoxal-*P* and concentrated with aquacide II.

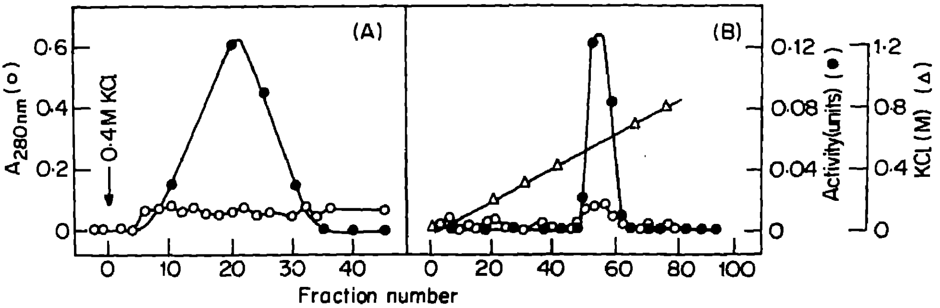

Figure 2. Chromatography of the DEAE-Sephadex eluate on diaminopimelate-Sepharose

(**A**). 32.5 ml of concentrated DEAE-Sephadex eluate, containing 16.3 mg protein and 58.8 units of activity, was adjusted to 0.2 M KCl and loaded onto a column (1×23 cm) of diaminopimelate-Sepharose equilibrated with buffer PM containing 0.2 M KCl. The column was washed with the equilibration buffer and then, buffer PM containing 0.3 M KCl, until the absorbance at 280 nm was negligible. The column was subsequently developed with buffer PM containing 0.4 M KCl. The flow rate was 36 ml per hour. Fractions 10-30 were pooled.

(**B**). Rechromatography on diaminopimelate-Sepharose. 129 ml eluate from the previous step (4.77 mg protein, 112 units activity) were loaded on a bed (1×23 cm) of diaminopimelate-Sepharose equlibrated with buffer PM. After washing the column, a linear gradient of KCl in buffer PM was applied. The two chambers of the gradient apparatus contained 100 ml of buffer PM and buffer PM containing 1 M KCl respectively. The flow rate was 24 ml per hour. Fractions 50-61 were pooled. Symbols: (O) protein (●) activity: (△) KCl gradient.

Aliquots (2.5 ml or less) of the concentrated enzyme were chromatographed on a 1.5×138 cm bed of Sephadex G-200 equilibrated with buffer PM containing 5 μM pyridoxal-*P*. Fractions with enzymatic activity were pooled. The results of the purification are shown in table 1.

Table 1. Purification of diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059.

Fraction	Total activity[a] (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Recovery (%)
Crude extract	316	5408	0.06	1	100
Streptomycin sulphate, 2%	266	4587	0.06	1	84
$(NH_4)_2SO_4$, 45-60%	205	3098	0.07	1	65
DEAE-Sephadex	127	35	3.62	61	40
Diaminopimelate-sepharose	114	4.84	23.50	398	36
Diaminopimelate-Sepharose rechromatographed	42	2.07	20.50	347	13
Sephadex G-200	34	1.58	21.40	363	11

Wet-packed cells (145 g) was used to prepare the crude extract.

[a] The enzyme was assayed with (DL+*meso*)-diaminopimelate containing 53% of the *meso*-isomer. The specific activity of the purified preparation was 30.60 units/mg protein when *meso*-diaminopimelate was used.

Homogeneity

Polyacrylamide gel electrophoresis of fraction 7 at pH 7.0 in the presence or absence of 5 μM pyridoxal-*P* revealed four protein bands, all of which had the decarboxylase activity. At pH 9.0, five protein bands were observed. One prominent band (molecular weight 100,000) and three very minor bands (molecular weights 45,000; 50,000; 150,000) were observed on SDS-polyacrylamide gel electrophoresis (figure 3). A single protein band corresponding to pI 3.6 was observed on isoelectric focussing. These data indicate that the enzyme preparation was homogeneous.

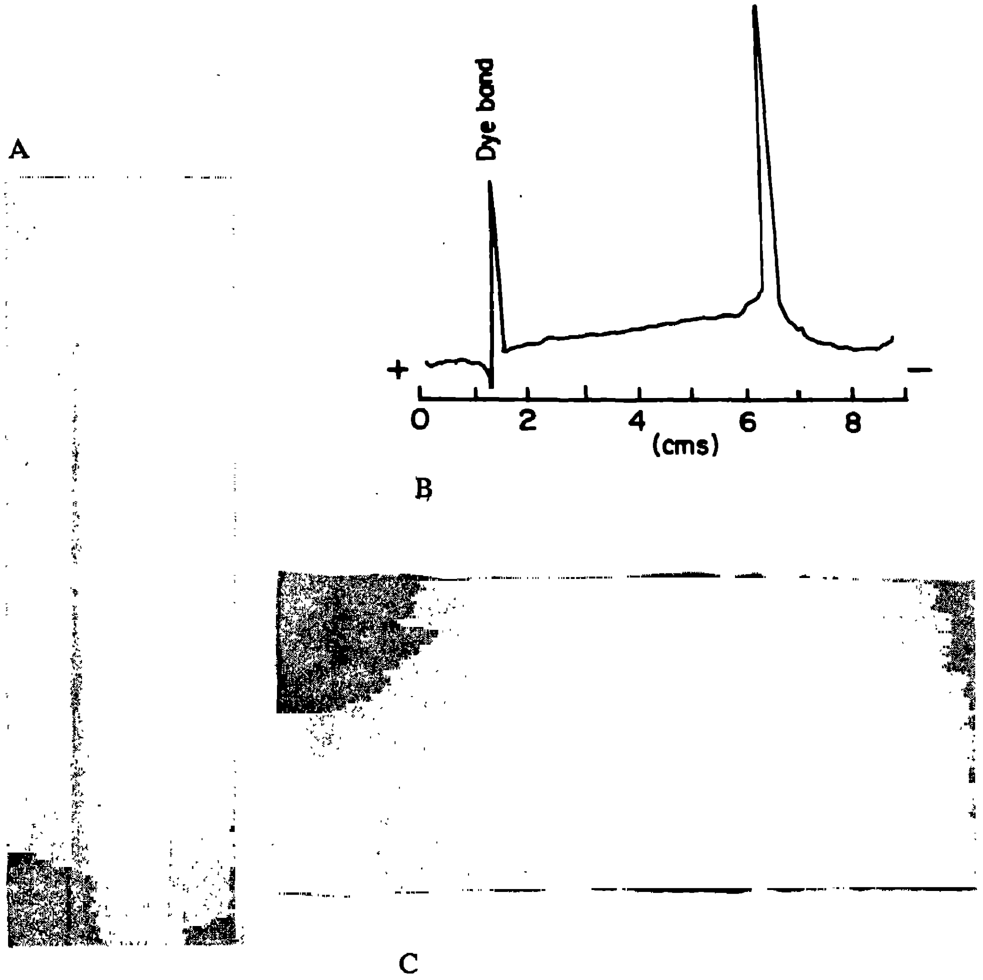

Figure 3. Electrophoresis of purified diaminopimelate decarboxylase. (A) SDS-polyacrylamide gel electrophoresis protein (50 μg) in a 10% gel. (B) A densitometer scan of the SDS-PAGE gel. (C) Isoelectric focussing of enzyme (75 μg) in the pH range 3-10. 1M NaOH and 1N H_3PO_4 were used as the cathode and anode wetting solutions. Electrophoresis was carried out at 8-9°C with a constant voltage of 400 V.

Conditions for optimal activity and stability

Diaminopimelate decarboxylase loses all activity upon freezing. In buffer P containing 5 µM pyridoxal phosphate and 0.4 M KCl, the enzyme did not loose a activity in twelve weeks at 4°C. By themselves, pyridoxal phosphate and K were not equally effective in stabilizing the enzyme. Unless mentioned otherwis the enzyme stored in the presence of pyridoxal phosphate and KCl was dialyze against buffer PM when used for the following experiments. This preparatio looses 25% activity in two weeks at 4°C.

The enzyme required sulphydryl groups for activity and stability. Added thio were not essential for activity (table 2). However dithiothreitol, 2,3-dimercapto propan-l-ol and reduced glutathione stimulated activity. Inhibition by sulphydr blocking reagents such as *p*-hydroxymercuribenzoate, iodoacetamide and N-ethy maleimide was reserved by an excess of thiols (table 3). Heavy metal ions such a Hg^{2+} (1 mM) and Cu^{2+} (1 mM) and Zn^{2+} inhibited the enzyme completely b (100%) by forming mercaptides. The inhibitions by Hg^{2+} and Cu^{2+} ions wer reversed by 2-mercaptoethanol (2 mM) to the extent of 42% and 69% respectively The inhibition by Zn^{2+} ion was not only reversed completely by dithiothreitol (mM) but the enzyme was also activated by 40%.

Table 2. The stability of the enzyme and the requirement for sulphydryl groups.

Time (days)	Specific activity (units/mg)			
	Experimental[a]		Control[b]	
	Without additions	+ Dithio-threitol (2mM)	Without additions	+ Dithio-threitol (2mM)
0	32.2	31.5	30.7	48.1
2	27.2	29.9	31.5	51.2
7	11.4	17.3	30.7	46.5
15	0.0	5.1	23.7	32.8

[a] The purified enzyme (33 µg) in buffer PM (1 ml) were loaded on a column (1×23.5 cm) of Sephadex G-25 which had been equilibrated in 0.1 M potassium phosphate buffer, pH 7.5. The column was developed with the equilibration buffer. The material collected in the void volume was used as a source of enzyme. Pyridoxal-P was added only when the enzyme samples were assayed.

[b] The control was an aliquot of the enzyme sample loaded on the column.

The enzyme samples were stored at 4°C.

The enzyme had optimal activity at pH 7.4-7.6 in 0.1 M Tris-HCl buffer, at pH 7-7.1 in 0.1 M imidazole-HCl buffer, and at pH 7.5 in 0.1 M potassium phosphate buffer.

Table 3. Effect of thiols and sulphydryl-blocking reagents on enzyme activity.

Additions	Activity (%)
None	100
2-mercaptoethanol, 2 mM	99
Glutathione, 2 mM	126
2,3-dimercaptopropan-1-ol, 1 mM	130
Dithiothreitol (DTT) (1 to 10 mM)	135
p-hydroxymercuribenzoate (pHMB), 1 mM	100
pHMB, 1.7 mM	24
pHMB, 1.7 mM+DTT, 7 mM	137
pHMB, 5 mM	0
pHMB. 5 mM (IAM), 7 mM	0
Iodoacetamide ((AM), 1 mM	87
IAM, 2 mM	53
IAM, 2 mM+DTT, 5 mM	92
IAM, 5 mM	8
IAM, 5 mM+DTT, 5 mM	63
N-Ethylmaleimide (NEM), 1 mM	101
NEM, 5 mM	17
NEM, 5 mM+DTT, 5 mM	44

The assay system (final volume 1ml) containing Tris-HCl buffer pH 7.5, 100 µmol; pyrido phosphate, 10 nmol; enzyme 1 unit and the test compound was preincubated at 37°C for 5 n before starting the reaction with *meso*-diaminopimelate, 5 µmol.

For checking the reversal of inhibition, after the enzyme was allowed to react with t compound for 5 min, thiol was added. The reaction mixture was incubated further for atlea min at 37°C, before starting the reaction with diaminopimelate.

Substrate specificity and concentration

The enzyme did not decarboxylate LL-diaminopimelate, L-lysine, D-lysine, I lysine, S-(2-aminoethyl)-L-cysteine or L-2-amino-pimelate (10 mM each), wh *meso*-diaminopimelate (5mM) was 64% decarboxylated. From a double recip cal plot of the substrate concentration and reaction rate (figure 4), the K_m *meso*-diaminopimelate was found to be 0.5 mM. The enzyme preparation was f of the racemase (Work, 1962) activity.

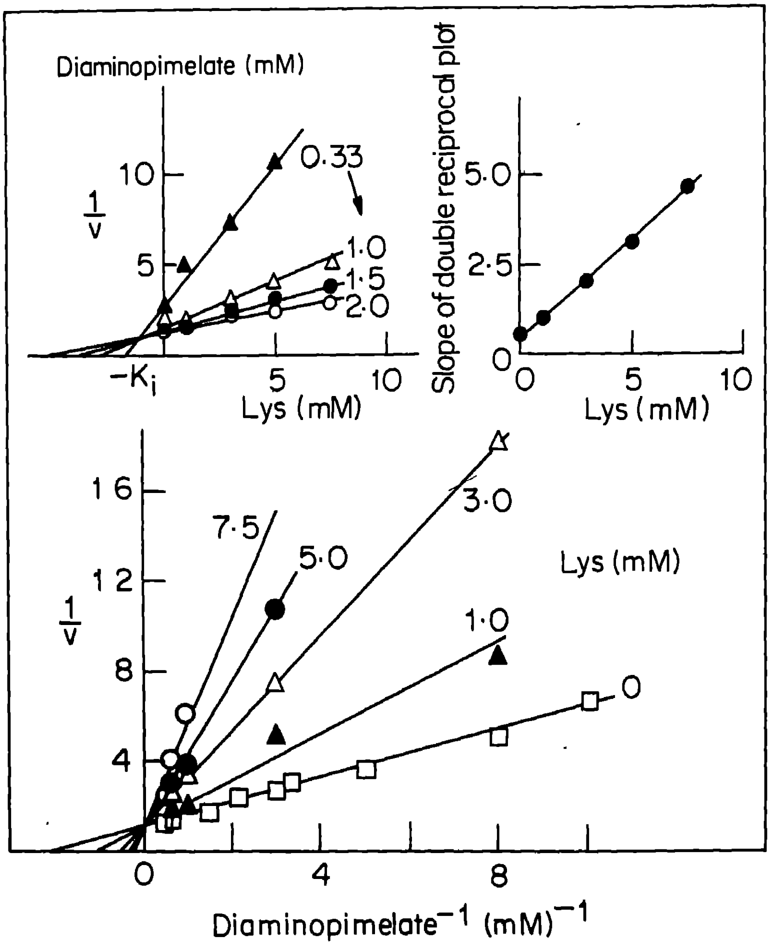

Figure 4. Inhibition of diaminopimelate decarboxylase by L-lysine with respect to diamin pimelate. Enzyme, (0.132 μg) was assayed with diamino [1,7−^{14}C] pimelic acid (A$_2$pm) c specified activity 500 μCi per mol. Velocity of the reaction is expressed as the amount (in cp of ^{14}CO$_2$ released in 10 min.

Cofactor requirement and concentration

When the DEAE-Sephadex eluate (Fraction 4, table 1) was assayed colorimetricall in the absence of added pyridoxal-*P*, no activity was detected. At this stage of purification, less than 1% of the maximal activity could be detected in the absence o added pyridoxal-*P* using the more sensitive radiochemical assay. When the purified enzyme had been stored in buffers containing pyridoxal-*P* it was not possible t remove bound pyridoxal-*P* merely by dialysis against buffer PM. In such cases, th enzyme was treated with hydroxylamine (Materials and Methods). The resultan enzyme preparation had about 3% of maximal activity in the absence of adde pyridoxal-phosphate, when assayed radiochemically.

The K_m for pyridoxal phosphate of the 'resolved' enzyme as determined by a double reciprocal plot of pyridoxal-phosphate concentration against reaction rate was 0.6 μM (figure 5). The enzyme was inactive with pyridoxamine-5'-phosphate or pyridoxal. Pyridoxamine-5'-phosphate inhibited the activity and this was reversed by an excess of pyridoxal-*P*.

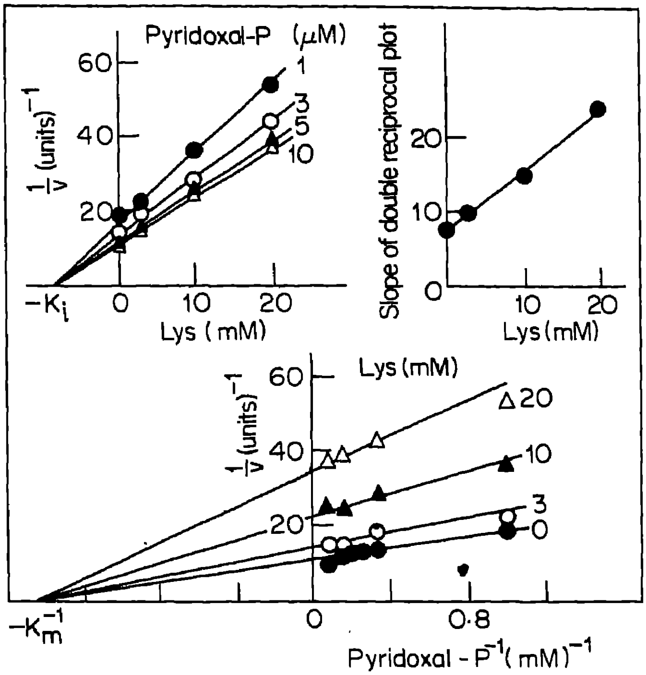

Figure 5. Inhibition of diaminopimelate decarboxylase by L-lysine with respect to pyridoxal-*P*. Three μg of 'resolved' enzyme was used for assay.

Effect of amino acids

Of the protein amino acids, only L-lysine inhibited the enzyme significantly and DL-aspartate-β-semialdehyde did so to a lesser extent at saturating and lower concentrations of diaminopimelate (table 4). The other amino acids tested had no effect. Glutaraldehyde at the same concentrations did not inhibit the enzyme when the concentration of *meso*-diaminopimelate was 5 mM.

Inhibition by L-lysine with respect to *meso*-diaminopimelate fell into the class termed as 'single site pure-competitive' [(figure 4) (Segel, 1975)]. The K_i for lysine as determined by the Dixon plot was 1.2 mM. Inhibition by L-lysine was 'single site pure non-competitive' (Segel, 1975) with respect to pyridoxal phosphate the K_i for lysine was 9 mM (figure 5). Lysine inhibition could not be dissociated from activity by altering the pH of the assay by heating the enzyme at 50°C for 15 min or by assaying the presence of a partially (50%) inhibitory concentration of iodoaceta-

Table 4. Effect of amino acids on diaminopimelate decarboxylase activity.

Additions (10 mM)	Activity (%) Diaminopimelate (0.6 mM)[a]	(5 mM)[b]
None	100	100
L-Pro	136	113[c]
L-Cys	81	86[c]
L-Arg	122	94
L-His	73	109
L-Lys	20	50
L-Thr	127	108
L-Met	65	108
L-Ile	109	102
L-Lys + L-Thr + L-Met + L-Ile	21	55
L-Leu + L-Val + L-Ile	81	97
L-Lys + L-Leu + L-Val + L-Ile	17	56
DL-aspartic-β-semialdehyde[d]	72	66

[a] Diamino [1,7−^{14}C] pimelate of specific activity 500 μCi per mol and 0.132 μg enzyme were used. The activity of the control sample was 5,500 cpm $^{14}CO_2$ released under standard assay conditions.

[b] The enzyme was assayed colorimetrically. 3.3 μg enzyme was used per assay.

[c] Assayed radiochemically with diamino [1,7−^{14}C] pimelic acid of specific activity 0.067 μCi per mol. The activity of the control was 5,200 cpm $^{14}CO_2$ released. 1.32 μg protein was used per assay.

[d] The concentration of DL-aspartic-β-semialdehyde was 1 and 2 mM respectively.

mide. These procedures were for dissociating activity from lysine inhibition—i.e. for desensitizing the enzyme if two sites are involved. Since lysine, a product of the reaction catalyzed by the decarboxylase, is a linear competitive inhibitor as evidenced by linear slopes replot (figure 5), it could be inferred that there are no additional sites for lysine binding other than the substrate binding site(s).

Inhibition by analogues of diaminopimelate

Structural analogues of diaminopimelate which were found to inhibit the decarboxylase are listed in table 5. D-Lysine, S-(2-aminoethyl)-L-cysteine, L-2, 4-diaminobutyrate, L-2-3-diaminopropionate, DL-2-aminoadipate, hexamethylenediamine, adipic acid, 6-aminohexanoic acid or glutaric acid (10 mM) had no effect at either concentration (0.6 mM or 5 mM) of diaminopimelate. It appears from these results that the best inhibitors are those that have two amino groups separated by five methylene groups. The efficacy of inhibition is increased by the presence of a carboxyl substituent in position 1 in the L-configuration.

Table 5. Inhibition of diaminopimelate decarboxylase activity analogues of diaminopimelate.

Additions (10 mM)	Activity (%)	
	Diaminopimelate[a] (0.6 mM)	Diaminopimelate[b] (5 mM)
None	100	100
(DL+*allo*)-Cystathionine	75	113
LL-Diaminopimelate	0.4	0.5[c]
LL-Cystine	58	83
L-Lysine methylester	31	81
Cadaverine	40	63
Putrescine	—	85
DL-2-Aminopimelic acid	49	105
Pimelic acid	42	97
L-Norleucine	45	76

a, b, c As in table 4.

— denotes 'not determined'.

Effect of other compounds

At a saturating concentration (5 mM) of *meso*-diaminopimelate, UDP-glucose mM), UTP, UDP or UMP or a mixture of the three nucleotides (1 mM each), AT ADP, AMP or a mixture of the adenyl nucleotides (1 mM each) whether in the pr sence or absence of 1 mM $MgCl_2$ had no significant effect on enzyme activity. A non-saturating concentration (0.6 mM) of *meso*-diaminopimelate, 47% activati was seen with a mixture of the adenyl nucleotides (1 mM each).

Molecular weight and subunit structure

The molecular weight of the enzyme was found to be concentration- and pyridox *P* dependent (table 6). The molecular weight of the major polypeptide cha

Table 6. Molecular weight of diaminopimelate decarboxylase.

Conditions of chromatography		Approximate molecular weight
Sample loaded (µg)	Buffer used	
100	PM	191,000
20	PM	191,000; 94,000
10	PM	94,000
10	PM containing 5 µM pyridoxal-*P*	191,000
100	PM containing 5 µM pyridoxal-*P*	191,000

One ml enzyme in buffer PM (Fraction 5 table 1) which had no detectable activity (by chem procedure) in the absence of added pyridoxal-*P*, was chromatographed on a calibrated be Sephadex G-200 (Methods). Diaminopimelate decarboxylase activity was monitored in eluate. The difference in elution volume between the two molecular weight species was 17 observed by sodium dodecyl sulphate polyacrylamide gel electrophoresis was 100,000. It was inferred from these data that the decarboxylase was composed of two subunits of approximate molecular weight 100,000. Dimerization was induced by pyridoxal phosphate and higher protein concentrations. These data also suggest that the minor bands of molecular weight 45,000, 50,000 and 150,000 observed on electrophoresis may be fragments of the 94,000 and 191,000 molecular weight species which arose by incomplete reduction or dissociation or by proteolytic cleavage during electrophoresis.

Discussion

Diaminopimelate decarboxylase has been obtained from *M. glutamicus* ATCC 13059 in a homogeneous form. Other homogeneous enzymes are known to exhibit multiple enzymatically active bands on polyacrylamide gel electrophoresis (due to association-dissociation) and move as a single band on SDS-polyacrylamide gels like the decarboxylase of *M. glutamicus* ATCC 13059 (Kleinshmidt and Kleiner, 1978; Feldberg and Datta, 1971). This is the first report of a homogeneous preparation of the enzyme. The enzyme from *M. glutamicus* ATCC 13286 showed four bands on polyacrylamide gel electrophoresis at pH 7.0 of which only one band had enzyme activity (Vitale *et al.*, 1977). The specific activity of the enzyme purified from strain ATCC 13059 was about one-and-a-half times higher than that reported for the purified enzyme from strain ATCC 13286.

The procedure for the purification of the decarboxylase of *M. glutamicus* ATCC 13059 described in this work differs from those described earlier in the use of an affinity column. Immobilized pyridoxal-phosphate derivatives (Fukui *et al.*, 1975) were not useful, although the enzyme that was chromatographed had an absolute requirement of pyridoxal-*P* for activity. The decarboxylase from *M. glutamicus* ATCC 13059 resembles the enzyme from several other sources in its specificity for *meso*-diaminopimelate (White and Kelly, 1965; Dewey *et al.*, 1954; Shimura and Vogel, 1966; Vogel and Hirvonen, 1971) and its requirement of pyridoxal-phosphate (White and Kelly, 1975; Norton and Chen, 1970; White, 1971; Dewey *et al.*, 1954; Rosner, 1975; Grandgenett and Stahly, 1968) and of Sulphydryl groups (White and Kelly, 1965; Vitale *et al.*, 1977; Dewey *et al.*, 1954; Grandgenett and Stahly, 1968).

The enzyme required added sulphydryl groups for stability. The stimulation by certain dimercapto compounds probably indicates that some more free sulphydryls needed for activity are formed or these compounds are more efficient in protecting enzyme sulphydryl groups. The inhibition by heavy metal ions and p-hydroxymercuribenzoate and reversal by sulphydryl compounds are to be expected. At high concentrations of p-hydroxymercuribenzoate, the enzyme is perhaps denatured and no reactivation can occur. But the reversal of inhibition of N-ethylmaleimide and iodoacetamide by sulphydryl compounds is hard to explain. These reagents are presumed to combine irreversibly with enzyme-SH groups and treatment of these alkylated-S-enzymes is not expected to release the inhibition. Since the inhibition is released in this instance, it is possible that these reagents may bind other groups reversibly. There are other such examples in literature (Vijayalakshmi *et al.*, 1975). The K_m for *meso*-diaminopimelate of this enzyme is a little lower than values reported for the enzyme from other bacterial sourc (White and Kelly, 1965; Norton and Chen, 1970; Vitale *et al.*, 1977; Rosner, 197 Grandgenett and Stahly, 1968; Kagan *et al.*, 1971) and a little higher than the valu reported for the plant enzymes (Mazelis and Creveling, 1978; Shimura and Voge 1966; Vogel and Hirvonen, 1971; Mazelis *et al.*, 1976). The K_m for pyridoxal-*P* the diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059 was 0.6 μM a was lower than the values reported for other decarboxylases (Norton and Che 1970; Mazelis and Creveling, 1978; Rosner, 1975). The observations of Vitale *al.*, (1977) led them to infer that inhibition of the decarboxylase by substra analogues did not require both ends of the molecule to be polar; the contribution size and pI appeared to be more important. The best inhibitor by far of the enzyn from *M. glutamicus* was LL-diaminopimelate. The others were not very effectiv they were of a similar size as the substrate and with both ends polar excepting norleucine which inhibited equally well. The effects of diaminopimelate analogu on the enzyme from various sources (White and Kelly, 1965; Dewey *et al.*, 195 Rosner, 1975) are all slightly different from one another and from the effects on t enzyme from the two strains of *M. glutamicus*. These differences may be in pa due to difference in assay conditions. The decarboxylases from most sources i cluding the enzyme from *M. glutamicus* ATCC 13059 are inhibited by L-lysi (White and Kelly, 1965; Vitale *et al.*, 1977; Rosner, 1975; Grandgenett and Stahl 1968); lysine inhibition is competitive with respect to *meso*-diaminopimelate. T enzyme from *M. glutamicus* ATCC 13059 was inhibited significantly by aspartic- semialdehyde (at 2 mM) and much more strongly by LL-diaminopimelate (at mM). Although several other amino acids inhibited or activated the enzyme (2 40%) at a non-saturating concentration of *meso*-diaminopimelate, these effec were not evident when higher concentrations of this compound were used. Sin LL-diaminopimelate does not appear to be an obligatory/free intermediate lysine biosynthesis in *M. glutamicus* (Leadlay, 1979), only the inhibitions by lysine and aspartic-β-semialdehyde may be of physiological significance.

The molecular weight of the diaminopimelate decarboxylase from the tv strains of *M. glutamicus* differ considerably. The enzyme from *M. glutamic* ATCC 13286 had an approximate molecular weight of 53,000 (Vitale *et al.*, 1977) determined by gel filtration. The enzyme from *M. glutamicus* ATCC 13059 consis of two polypeptide chains of approximate molecular weight 100,000. The enzyn from *Bacillus subtilis* (Grandgenett and Stahly, 1971) and *E. coli* (White and Kell 1965) have approximate molecular weights of 105,000 and 200,000 respectivel Although many amino acid decarboxylases have a subunit structure, this is the fi report of the subunit structure of diaminopimelate decarboxylase. Associatio dissociation occurring at low protein concentrations such as those used in the experiments, has been reported for the chorismate mutase-prephenate dehydr tase of *Salmonella typhimurium* (Schmidt and Zalkin, 1971).

Thus, the diaminopimelate decarboxylase of *M. glutamicus* ATCC 13059 gros resembles most other diaminopimelate decarboxylases, although it differs from t enzyme of *M. glutamicus* ATCC 13286 in several aspects.

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Purification and physicochemical properties of α-amylase from irradiated wheat

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Abstract. α-Amylases from control and gamma-irradiated (at 0.2 and 2.0 kGy dose levels) wheat seedlings were purified to homogeneity and characterized. The molecular weight of the enzyme from a 2 kGy irradiated sample was slightly lower than that of the control; other general and catalytic properties also showed some differences. α-Amylase from the irradiated (2 kGy) sample had a narrow range of pH optimum and was inactivated faster at alkaline pH and by heat treatment than the enzyme from unirradiated wheat. A high apparent Michaelis constant (K_m) and a low maximal velocity (V_{max}) for the hydrolysis of soluble starch catalyzed by the enzyme from irradiated (2 kGy) wheat, suggested some modifications in the formation of the substrate α-amylase complex. Further, of the total number of amino acid residues lost on irradiation, dicarboxylic amino acids constituted the largest percentage; these structural alterations in the enzyme may be responsible for its partial inactivation. The total sugars liberated upon amylolysis of starch with the 2 kGy irradiated enzyme were lower than control, and there was accumulation of higher maltodextrins in the place of maltose.

Keywords. Wheat; α-amylase; γ-irradiation; purification; physicochemical properties.

Introduction

α-Amylase (EC 3.2.1.1) in wheat (Varner *et al.*, 1965) and barley (Daussant and Corvazier, 1970) has a decisive role in seedling growth. It was shown earlier (Ananthaswamy *et al.*, 1971) that the activity of α-amylase isolated from γ-irradiated wheat seedling was significantly lower than that in the control. This was attributed to a radiation-induced delay in the differentiation of the enzyme into its functional isoenzymic forms (Machaiah and Vakil, 1979). Prior treatment with ionizing radiation reduces the catalytic activities of catalase and lipase in safflower (Singh, 1974) and peroxidase isoenzymes in beans during germination (Habeck and Curtis, 1974). In the present investigation, α-amylases isolated from control and gamma-irradiated wheat seedlings, were partially purified and characterized. An attempt was made to correlate the partial inactivation of the enzyme to the structural modifications due to exposure of wheat to a high dose of radiation.

Materials and methods

Samples of 'CV, Vijay' variety of wheat were obtained from the Agricultural Research Station, Niphad, Maharashtra. The seeds were stabilized at 11%

moisture level, sealed in polyethylene bags and irradiated (at 0.2 and 2.0 kGy dose levels) in ^{60}Co-gamma chamber-900, with an influx of 2500 Ci and overdose ratio of 10%. The samples were stored at 25°C for 4 weeks before the commencement of germination tests. Glycogen (rabbit liver) was purchased from V.P. Chest Institute, New Delhi and Sephadex G-75 from Pharmacia, Uppsala, Sweden. Bio-Gel P-100 was obtained from Bio-Rad Laboratories, Richmond, California, USA. Other chemicals were from Sigma Chemical Co., St. Louis, Missouri, USA.

Germination

Control and irradiated wheat samples (5 g lots) were surface-sterilized with 1% sodium hypochlorite solution and imbibed with distilled water for 16 h. Incubation was carried out for 4 days at 25°C in dark on petri-dishes layered with filter paper, and moistened with streptomycin and penicillin (5 µg/ml), to check microbial growth.

Purification of α*-amylase*

The shoot-root axis and scutelum of 4-day old wheat seedlings (from 20 g wheat) were removed and the endosperms homogenized in chilled (3°C) 0.5 M Na-acetate buffer (pH 5.6) containing 0.003 M $CaCl_2$ in a precooled Waring blendor and centrifuged at 5,000 *g* for 10 min. The supernatant solution was adjusted to pH 8.0 with 1 N NaOH, heated at 70°C to inactivate β-amylase and centrifuged at 5,000 *g* for 10 min. α-Amylase was precipitated from the crude extract and isolated as enzyme-glycogen complex essentially as described by Loyter and Schramm (1962). Absolute ethanol (final concentration 40%) and 1.6% purified glycogen (0.2 ml/1000 units of the enzyme activity) were added to the cooled supernatant. After two washes with 40% ethanol, the precipitated glycogen-α-amylase complex was dispersed in 0.02 M Tris-HCl buffer (pH 8.2) containing 0.002 M $CaCl_2$, incubated for 1 h at 37°C to release the enzyme and centrifuged at 11,000 *g* for 10 min. Further purification of the enzyme was achieved by passing the supernatant through a Sephadex G-75 column (50×1 cm), pre-equilibrated with 0.02 M Tris-HCl buffer (pH 8.2), containing 0.002 M $CaCl_2$. Three ml fractions were collected and those having α-amylase activity were pooled, and precipitated with $(NH_4)_2SO_4$ (45% saturation). The precipitate, redissolved and dialyzed against the same buffer for 6 h at 25°C, was further subjected to chromatography on Bio-gel P-100 column (65×2.5 cm) and the protein was eluted with the same bufer. The fractions containing α-amylase activity were pooled and the enzyme protein was reprecipitated at 45% saturation of $(NH_4)_2SO_4$. The purified enzyme was dialyzed against the same buffer for 6 h and used for subsequent studies (table 1). The homogeneity of the enzyme was indicated by polyacrylamide gel electrophoresis. The zymogram showed three protein bands, all having α-amylase activity (data not shown). The same extent of purification and recovery were achieved with irradiated samples.

Protein estimation

Protein content was determined according to the method of Miller (1959) using bovine serum albumin as standard.

Table 1. Purification of α-amylase from germinating wheat.

Purification step	Total proteins (mg/g wheat)	Total activity (units/g wheat)	Sp. act. (units/mg protein)	Purification fold
Crude extract	42.00	5168	123	1
Heat-treated supernatant	17.00	4785	281	2
Glycogen complex	0.97	3970	4085	32
Sephadex G-75 fraction	0.78	3314	4400	37
Bio-gel P-100	0.49	2380	4900	40

α-*amylase activity*

The enzyme preparation (about 10 μg protein) was incubated with 0.5% starch and 0.01 M acetate buffer (pH 5.6) for 30 min at 37°C. The reaction was stopped by the addition of 5 ml of 0.5 N acetic acid. An aliquot of the reaction mixture was added to 10 ml of iodine reagent and the reduction in the colour was measured at 700 nm in a Bosch and Lomb Spectronic 20 colorimeter. Enzyme unit: The amount of enzyme required to produce a 10% decrease in absorbance at 700 nm in 30 min at 37°C and pH 5.6.

The enzyme (100 μg protein) was incubated in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulphate, 1% β-mercaptoethanol and 10 mM dithiothreitol at 37°C for 2 h. Electrophoresis was then performed in tubes (0.6×10 cm) at 25°C for 5 h in 10% polyacrylamide gel and 1% sodium dodecylsulphate, essentially as described by Robyt *et al.* (1971). The bands were stained for proteins with Coomassie Blue for 24 h. The molecular weight of α-amylase was determined by comparison of its relative electrophoretic mobility with that of bovine serum albumin (68,000), chymotrypsin (23,000) and myoglobin (17,000). The destained gels were scanned at 600 nm in a Canalco model F microdensitometer (1—control; 2—0.2 kGy; 3— 2.0 kGy).

Amino acid analysis

The sample (0.2 to 0.3 mg protein) was hydrolyzed with 6 N HCl in an evacuated sealed tube for 24 h at 110°C. Excess acid was removed *in vacuo* and the residue was taken in 0.2 M sodium citrate buffer (pH 2.2). Amino acid analyses were carried out with a Beckman-Spinco model 120 amino acid analyser (Schramm, 1954). To determine cysteine, an aliquot of the enzyme was oxidized with 1% performic acid prior to acid hydrolysis. Corrections for incomplete hydrolysis and decomposition of amino acids during heating were made (Tkachuk and Kruger, 1974). Tryptophan was estimated separately by the colourimetric method of Spies (1967). Nitrogen was determined by micro Kjeldahl method (Hawk, 1954).

Sugars in the hydrolysate were identified and quantified by descending paper chromatography in butanol-pyridine-water (6:4:3). Reducing sugars on the chromatograms were detected by alkaline silver nitrate spray (Trevelyan *et al.*, 1950).

Results

Total α*-amylase activity*

The specific activity of the enzyme from 4-day old irradiated (2 kGy) wheat seedlings was 180±15.3 units and was significantly low ($P<0.001$) compared to the value of 2380±38.3 units in the control. The activity of the enzyme in the seedlings irradiated with a lower dose was 2000 ±17.8.

pH optima

The enzymes isolated from control and 0.2 kGy irradiated samples, exhibited almost identical and broad pH optima, ranging from pH 3.6 to 5.6 (figure 1). The

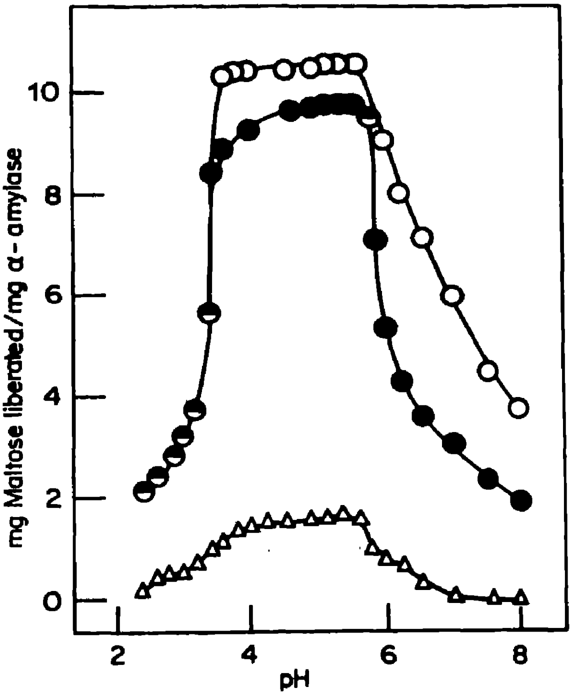

Figure 1. Effect of medium pH on the activity of α-amylase isolated from irradiated wheat seedlings. The pH optimum of α-amylase was determined using 0.02 M HCl-glycine (pH 2.4 to 3.4), 0.02 M Na acetate (pH 3.5 to 5.6) and 0.02 M Na phosphate (pH 5.8 to 8.0) buffers containing 0.001 M $CaCl_2$. The enzyme (10 μg) was added to the reaction mixture containing 2 ml each of the buffer and 0.5% starch and incubated at 37°C. After 10 min, 0.1 ml 5 N HCl was added and the mixture centrifuged (5000 *g* for 10 min). Reducing sugars in the filtrate were estimated (Somogy, 1952).
Control (O); 0.2 KGy (●); 2.0 KGy (Δ).

enzyme from wheat irradiated at higher dose (2 kGy) had a narrower range of pH optima (pH 4.6 to 5.6) and was inactivated at a much faster rate in alkaline medium. Stability of the enzyme expressed as per cent of original activity at the pH optimum of 5.6 was reduced at pH 6.0 by about 60% in the sample irradiated at 2 kGy, compared to only 15% in control. The enzyme was almost completely inactivated at pH 7.0 in the sample irradiated at higher dose, whereas 55% of the activity was retained in the control.

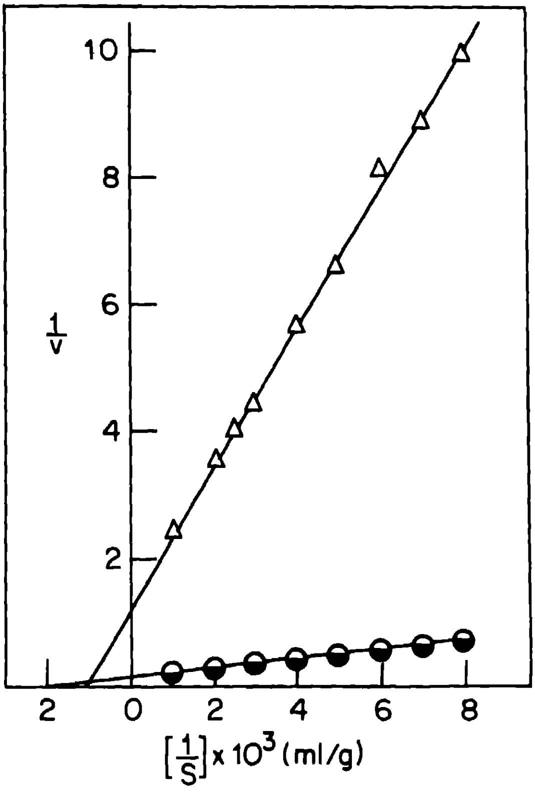

Figure 2. Effect of increasing substrate concentration on α-amylase isolated from control and irradiated wheat seedlings. The enzyme (10 μg) was incubated with 0.1 to 1 mg/ml of soluble starch buffered with 0.1 M Na acetate (pH 5.6, 0.003 M $CaCl_2$) at 37°C for 5 min. Control (O); 0.2 KGy (●); 2.0 KGy (Δ).

K_m value for soluble starch for α-amylase

Apparent K_m values for α-amylase, measured at pH 5.6 are shown in figure 2. The Lineweaver-Burk double reciprocal plots yielded straight lines giving comparable apparent K_m values for α-amylase from control (0.555 mg/ml) and 0.2 kGy (0.585 ±0.0048 mg/ml, $P<0.1$) samples. This value was however about 90% higher (0.95 ±0.026 mg/ml, $P<0.001$) for the enzyme from 2.0 kGy sample than that of control. Thus V_{max} (calculated as mg maltose released/5 min/mg enzyme at 37°C) was 7.0 and 0.77 for control and 2.0 kGy samples, respectively.

Effect of heat treatment

Although, α-amylase was not inactivated upto 70°C, at pH 8.0, the enzyme loses its activity, when subjected to higher temperature at pH 5.6 (figure 3). However, the rate of heat inactivation was faster with α-amylase isolated from the sample irradiated at 2 kGy compared to those from control and 0.2 kGy irradiated seedlings (figure 3). At 60°C, 76% of the original activity was retained after 30 min of heat treatment in control, whereas only 44% of the activity was recovered from the irradiated (2 kGy) sample.

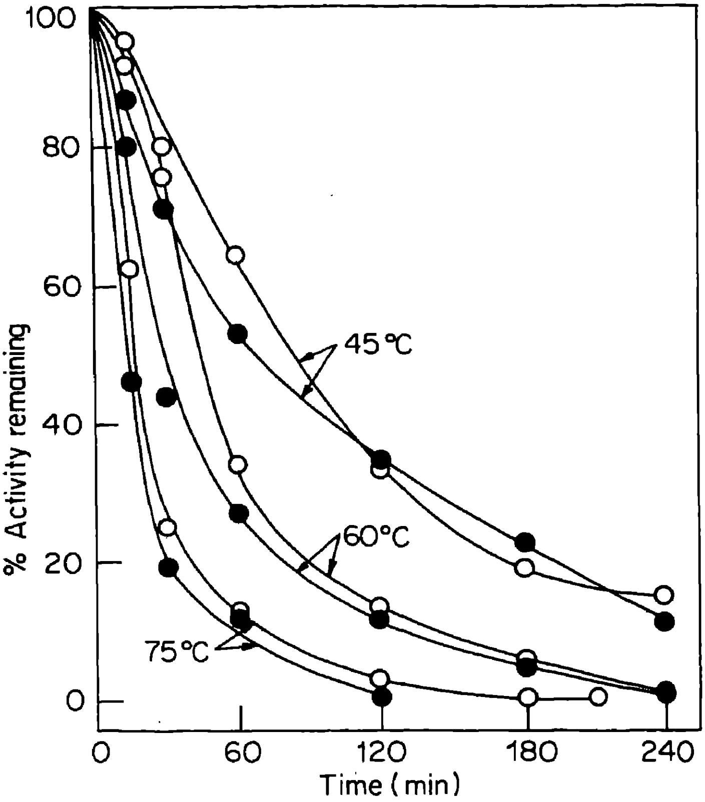

Figure 3. Effect of temperature on the activity of α-amylase. To measure the heat lability, the enzyme (10 μg) was incubated at temperatures ranging from 45 to 75°C for 0 to 4 h in 0.01 M Na acetate buffer (pH 5.6) containing 0.001 M $CaCl_2$.

The solutions were rapidly cooled and α-amylase activity was measured at 37°C. control (O); 2.0 kGy (●).

Isoelectric point

Isoelectric focusing (figure 4) of α-amylase on a gradient of pH from 4.0 to 7.0 resulted in the enzyme from the control sample eluting as a single peak in the range of 5.4 to 6.3 with a maximum at pH 5.8. Comparable results were obtained with the

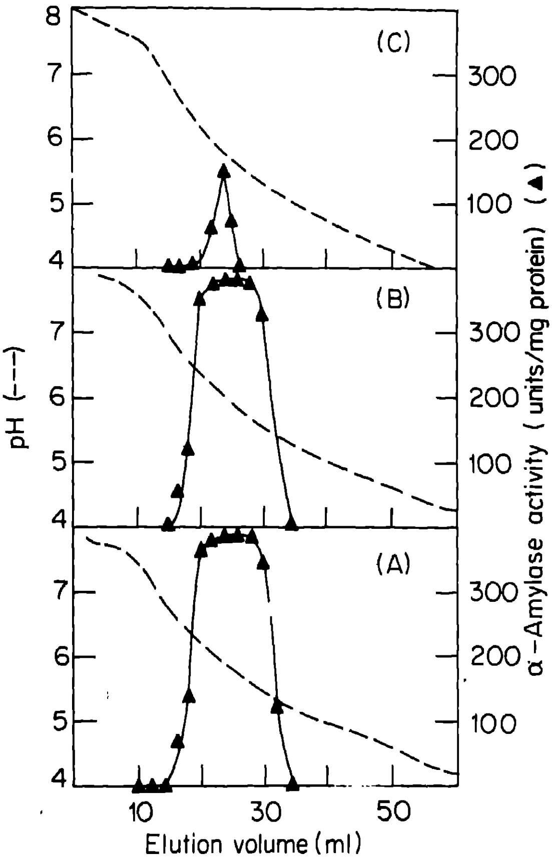

Figure 4. Isoelectric focusing of α-amylases, purified from control and irradiated wheat seedlings. α-amylase (200 μg) was electrofocused on an LKB 8101 column (110 ml) using 5 to 50% sucrose gradient and ampholines of pH from 4 to 7 as described by MacGregor (1978). After 66 h run, 1 ml fractions were collected from the column, and the enzyme activity and pH were determined in each fraction. A, control; B. 0.2 kGy; C, 2.0 kGy.

0.2 kGy irradiated sample. However, the enzyme isolated from wheat irradiated at 2 kGy eluted as a very narrow peak, in the range of pH 5.65 to 6.05 with a maximum at pH 5.8. This may be due to the total absence of the α-isoenzyme, having higher anodic mobility in the irradiated sample (Machaiah and Vakil, 1979).

The molecular weight of α-amylase was determined by gel filtration on a pre-equilibrated Bio-Gel P100 column (65×2.5 cm) using 0.02 M Tris-HCl buffer, pH 8.2 and eluted with the same buffer at a flow rate of 22 ml/h. Proteins of known molecular weights (given in paranthesis) albumin (68,000), pepsin (35,000) chymotrypsin (23,000) and myoglobin (17,000) were used to calibrate the column. The enzyme activity and A_{280} absorption were measured in each fraction (5 ml). The enzymes from control, 0.2 kGy and 2.0 kGy samples have molecular weights of 38,500, 38,000 and 37,000 respectively.

When subjected to polyacrylamide gel electrophoresis in the presence of sodi dodecylsulphate and β-mercaptoethanol the enzyme could be resolved into t distinct protein bands (figure 5) of apparent molecular weights of 40,000 (A_1) a 83,000 (A_2) daltons. Since both these bands had enzymic activity, though reduc considerably, it was presumed that wheat α-amylase could dimerise. Simi dimerisation of the enzyme from malted wheat on prolonged storage has be reported (Tkachuk and Kruger, 1974). The distribution pattern of the enzyme these bands indicated that in control, about 75% of the total activity was locali in the A_1 band and the rest in the A_2 band. In contrast, only 20% of the total amylase was in the A_2 band of the 2 kGy sample.

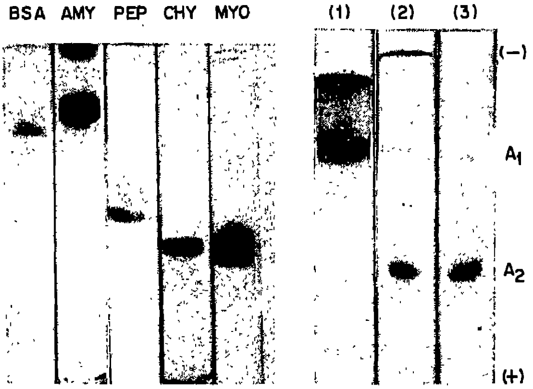

Figure 5. Determination of molecular weight of α-amylase by sodium dodecyl sulpl polyacrylamide gel electrophoresis. The enzyme (100 μg) was incubated in 0.01 M sod phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulphate 1% β-mercaptoethanol 10 mM dithiothretol at 37°C for 2 h. Electrophoresis was then performed in tubes (0.6×10 at 25°C for 5 h in 10% polyacrylamide gel and 1% sodium dodecyl sulphate, essentiall described by Robyt *et al.* (1971). The bands were stained for proteins with Coomassie Blue 24 h. The molecular weight of α-amylase was determined by comparison of its relative elec phoretic mobility with that of bovine serum albumin (68,000), chymotrypsin (23,000) and n globin (17,000). The destained gels were scanned at 600 nm in a Canalco model F microde tometer. 1—control; 2—0.2 kGy; 3—2.0 kGy.

Amino acid composition of α-*amylase*

The amino acid composition (table 2) shows that about 398, 386 and 373 amino a residues are present per molecule of α-amylase isolated from control, 0.2 and kGy samples, respectively. In the control, aspartic and glutamic acids constitu the 91 acidic amino acid residues, whereas lysine, hisitidine and argin constituted the 62 basic ones forming an excess of 29 cationic groups. I accurate calculation of the number of acidic and basic amino acid residues, NH_3 content is not taken into consideration. In α-amylase isolated from irradia (2 kGy) wheat seedlings the number of basic and acidic amino acid residues w

Table 2. Amino acid composition of purified α-amylase from control and irradiated wheat.

Amino acid	Control	0.2 kGy	2.0 kGy
		amino acid residues	
Lysine	19	17	14
Histidine	17	15	7
Arginine	26	25	10
Aspartic	31	30	22
Glutamic	60	56	40
Threonine	16	16	24
Serine	17	16	20
Tyrosine	10	10	13
Glycine	39	39	36
Alanine	26	26	32
Valine	26	27	28
Isoleucine	19	20	22
Leucine	36	36	38
Proline	26	24	35
Phenylalanine	16	15	16
Tryptophan	10	10	10
Methionine	3	3	5
Half cystine	1	1	1
Total number	398	386	373
Basic	62	57	31
Acidic	91	86	62
NH_3	52	46	33

Amino acid content was calculated as μmol per 100 g of protein and converted to the nearest integral number of residues. Each value represents the average of two estimations in duplicate.

reduced by 50 and 30% respectively. Though histidine residues were less by 40%, no significant differences in tyrosine and tryptophan contents were observed. Proline, alanine and serine contents were slightly higher in irradiated samples.

Action pattern of α-amylase on soluble starch

Resolution of the products of α-amylolysis of soluble starch by paper chromatography revealed that the hydrolysis took place in two distinct stages. In the initial stage (figure 6) a series of maltodextrins with low Rf values (G_5 to G_{10}) were predominant, whereas in the second stage (figure 6B), maltose (G_2) and maltotriose (G_3) appeared in appreciable quantities and higher maltodextrins (G_8 to G_{10}) disappeared. The Rf value of glucose was taken as 1.0 and those of other sugars were calculated as: G_2=0.65; G_3=0.42; G_4=0.25; $G_5$0.16; G_6=0.10 and G_7 = 0.06. Achroic point with iodine was reached at 5 h of hydrolysis. The dextrin pattern remained stable up to 24 h of hydrolysis, but only traces of glucose were found after 3 h of hydrolysis. These observations are characteristic of cereal α-amylase (MacGregor *et al.*, 1971).

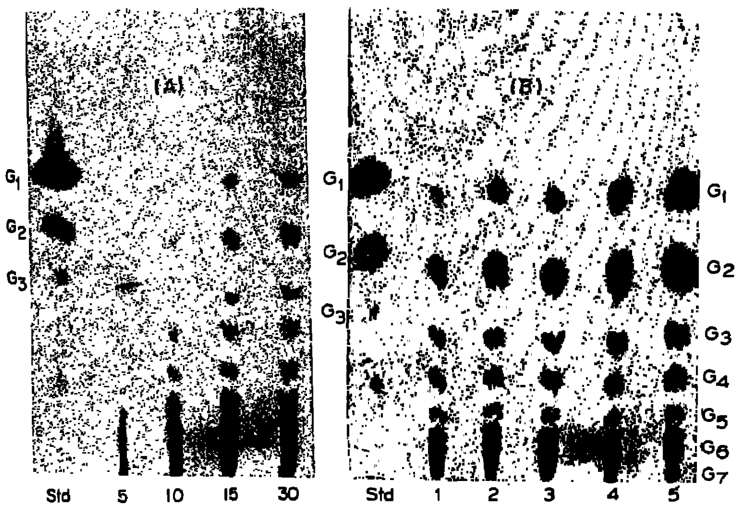

Figure 6. Action pattern of α-amylase (control A) on soluble starch. The purified enzyme (1 μg) isolated from control and irradiated wheat seedlings, was disolved in 0.1 M acetate buffe pH 5.6 containing 1 ml of 2% starch and incubated at 25°C. Products of hydrolysis in th filtrate were separated by descending paper chromatography. G_1—glucose, G_2—maltos G_3—maltotriose; G_4—maltotetrose; G_5—maltopentose; G_6—maltohexose; G_7—maltoheptos The reaction was stopped by immersing the tube in boiling water for 2-3 min, cooled, an centrifuged (5000 *g* for 10 min). Total sugar in the filtrate was determined by the phenol-H_2SO_4 method as described by Dubois *et al.* (1956) for 5, 10, 15 and 30 min P and A; for 1, 2, 3, and 5 h, P and B.

Quantitative yield of hydrolytic products of α-amylolysis

The rates of hydrolysis of starch, catalyzed by the α-amylases isolated from contro and irradiated wheat seedlings, were measured and compared at constan substrate: enzyme ratio and at achroic point (5 h at 25°C). Maltodextrin produced were analyzed quantitatively. It is seen (table 3) that the amount o reducing sugars produced by α-amylase from the 2 kGy irradiated sample wa significantly low ($P<0.001$), indicating that its hydrolyzing capacity was reduced After 5 h of hydrolysis, glucose and the lower maltodextrins (G_2 to G_4) constitute only 27% of the total hydrolytic products produced by the irradiated sampl compared to 44% by the control α-amylase. Thus, initial cleavage product representing G_6 and G_7 were predominantly present in the irradiated sample (kGy).

Discussion

It is clear from these studies that the irradiated ion at low dose (0.2 kGy) had n appreciable effect on some physicochemical properties of α-amylase. However the narrow range of pH optima, rapid inactivation in alkaline pH and higher hea lability of the enzyme isolated from the 2 kGy irradiated sample suggested that it stability was severely affected by a high dose of radiation. The very low biologica activity of α-amylase from the 2 kGy irradiated sample can be attributed to th absence of the α_1-isoenzyme, the most ionized isoenzyme at alkaline pH

Table 3. Quantitative yields of maltodextrins by action of α-amylase from irradiated whe seedlings.

		Control		0.2 kGy		2.0 kGy	
Sugar spots	Rf value of sugar	Reducing sugar (μg)	% of total	Reducing sugar (μg)	% of total	Reducing sugar (μg)	% of total
G_1	1.00	20.0	3.90	17.5	3.54	8.0	4.06
G_2	0.65	131.2	25.68	124.0	25.05	35.0	17.77
G_3	0.42	38.0	7.44	30.0	6.10	7.2	3.68
G_4	0.25	37.2	7.28	22.0	4.44	4.2	2.16
G_5	0.16	20.0	3.90	13.0	2.63	1.0	0.05
G_6	0.10	128.0	25.05	145.0	29.29	38.7	19.67
G_7	0.06	84.6	16.56	94.0	18.99	71.7	36.42
Origin	0.00	51.6	10.10	51.0	10.30	31.0	15.74
Total	—	510.6 ±10.82	100.0	496.5 ±5.8[a]	100.0	196.8 ±4.09[b]	100.0

The purified enzyme (10 μg protein), isolated from control or irradiated wheat seedlings, wa incubated with 1% starch for 5 h. The spots corresponding to glucose and each maltodextri (G_2 to G_2) were cut from the undeveloped chromatogram (figure 6), eluted with distilled wate and estimated for reducing sugars. Results are expressed as % of total and the values ar average of four experiments, in duplicate. [a]$P<0.01$, [b]$P<0.001$.

Nishikawa and Nobuhara (1971) have shown that at least two triplicate geneti systems control different α-amylase isoenzymes in wheat, and these are in very clos functional relationship with one another. Radiosensitivity of α-amylase synthesi depends upon the genome functional state (Bulakh and Grodzinky, 1974). Thus the low enzyme activity in irradiated sample may be due to more radiation damag of D genome, controlling the synthesis of α_1-isoenzyme (Nishikawa *et al.*, 1975) The elution of α-amylase as a narrow peak during isoelectric focussing (figure 4 also indicates some modifications in the polypeptide chain of the enzyme in the kGy irradiated sample. The high K_m value and change in $V_{\max}$ in the irradiate sample (figure 2) also indicate a much different protein.

Further, the catalytic abilities of α-amylases were determined in analyzing th hydrolytic products formed. The results (table 3) indicate that the enzym isolated from irradiated (2 kGy) wheat was less effective in attacking starc molecules resulting in the accumulation of sugars higher than maltoheptose (G_7) In all the groups, the enzyme hydrolyzed maltose to glucose at a much slower rat (table 3). The G_2/G_1 ratio has been calculated as 6.5 and 4.3 in control an irradiated samples, respectively. Thus, the enzyme isolated from irradiated whea (2 kGy) was less efficient in attacking higher molecular weight maltodextrins However, it should be stressed that the enzyme activity was not completel destroyed and the characteristic fractionation pattern of starch to oligosaccharides was not altered by radiation treatment. Nishikawa *et al.* (1978) have presumed tha the functional differentiation of starch degrading activity of the two groups of α-amylases, can be attributed to the differences in the content of their catalyticall active isoenzymes. The foregoing results suggest that treatment with high dose o radiation affects the general and catalytic properties of α-amylase isolated from germinating wheat.

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In vitro and *in vivo* nitrogenase activity of *Rhizobium* mutants and their symbiotic effectivity

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Abstract. A slow growing nitrogen-fixing strain of *Vigna radiata* var. *aureus* (mung bean) *Rhizobium* which expressed nitrogenase activity in a synthetic medium was isolated from its native population. Mutants with decreased and increased nitrogenase activity were derived from this strain by treatment with acridine orange and ethidium bromide. These mutants were tested for symbiotic effectivity *in vivo*. The effectivity of mutants with decreased nitrogenase activity in the culture medium was lower than the parent strain; however, the effectivity of mutants with higher nitrogenase activity did not increase above that of the parent. This suggests that the plant is perhaps a limiting factor in the full expression of rhizobial nitrogenase in the nodules.

Keywords. Nitrogenase activity; symbiotic effectivity; inoculation; mutant strains; nodulation; nitrogen fixation.

Introduction

In legume-*Rhizobium* symbioses, variabilities in symbiotic effectiveness which are either due to variations in nitrogen fixing potential of *Rhizobium* strains (Brockwell *et al.*, 1968; Chatel and Parker, 1973; Dadarwal *et al.*, 1974, 1979) or due to host genotypic compatibility (Nutman, 1969; Brockwell and Katznelson, 1976), are often observed. Since the nitrogen-fixing genes are known to be confined to the bacterial genome (Gibson, *et al.*, 1976), the host effects are mainly on symbiotic establishment and on controlling the induction and expression of nitrogenase in the nodules. The variability in the effectiveness of native *Rhizobium* isolates even on a single cultivar of a legume crop, gives the impression that the nitrogen-fixing ability of *Rhizobium* could be improved either by strain selection or by genetic manipulation. Since the nitrogen-fixing ability is expressed only in symbiotic association, it is not possible to find out whether the restriction on the bacterial gene expression is due to the bacterial genome or the plant. Recently, methods for inducing the nitrogenase under culture conditions in some slow growing *Rhizobium* strains have been reported (Child and Kurtz, 1978; Wilcockson and Werner, 1978; Kennedy and Pankhurst 1979). Having standardized the conditions for the expression of nitrogenase in a culture medium by a mung bean-*Rhizobium* strain, S24, we have derived mutants with increased and decreased nitrogenase activity as compared with the parent strain. In this paper, we report the symbiotic behaviour of these mutants in relation to the parent strain.

Materials and methods

The *Rhizobium* strain, S24, was isolated originally from the root nodules of mung bean (*Vigna radiata* var. *aureus*) cvr. Varsha. The strain, maintained on yeast extract-mannitol-agar (Vincent, 1970), was inoculated into 50 ml of filtered yeast extract-mannitol broth in 150 ml capacity flasks containing 10 µg/ml of acridine orange and ethidium bromide in separate flasks. The flasks were incubated on a shaker for 4 days at 28 ±1°C, subcultured in the same broth with added mutagens and incubated for a further 6 days. The broth culture was then diluted and plated on yeast extract-mannitol-agar medium plates and the plates were incubated for 10 days at 28 ±1°C. Colonies that appeared on the plates were selected and screened for nitrogenase activity on yeast-extract-mannitol-glutamate-succinate-agar slants (g/L:yeast extract, 0.2; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCO_3$, 0.5; mannitol, 10; sodium glutamate, 0.32; sodium succinate, 1.6; agar 15; pH 7.2). The parent S24 strain showed high nitrogenase activity when grown on this medium. The procedure for determining nitrogenase activity on agar slants and its interpretation is the same as described earlier (Dadarwal *et al.*, 1980).

Selected mutant clones with higher and lower nitrogenase activity than the parent strain on yeast extract-mannitol-glutamate-succinate-agar medium, were further tested for their nitrogenase activity on media containing sugars, amino acids and citric acid cycle intermediates in different combinations.

Nodulation and nitrogen fixation

Four selected mutants were tested along with the parent strain for nodulation and nitrogen-fixing ability on mung bean var. K851. Surface sterilized seeds were inoculated with 4-day old broth culture (5×10^5 to 10^6 viable cells/seed) and sown in sterilized Leonard jars containing Sloger's N_2-free mineral salt solution (Sloger, 1969). Uninoculated seeds were sown as controls. Nodulation, nodule nitrogenase activity, plant weight and plant nitrogen content were determined at 15, 25 and 35 days of plant growth. Nitrogenase activity in intact nodules was determined by gas chromatography using the C_2H_2 reduction technique of Hardy *et al.* (1968). The plants were dried, weighed and the plant nitrogen determined by the conventional micro-Kjeldahl method. The ratios of plant weight and plant nitrogen were then calculated from control values.

The parent strain S24 has an electron recycling hydrogenase system (Dadarwal *et al.*, 1980). The hydrogenase in intact nodules of all the strains was also determined indirectly by determining the C_2H_2 reduction rates, both in the presence and absence of 0.1 atm. hydrogen (Emerich *et al.*, 1979).

Results

Selection of mutants with increased or decreased nitrogenase activity

A total of 2,000 clones were screened for nitrogenase activity on yeast extract-mannitol-glutamate-succinate-agar medium. All the clones obtained had nitrogenase activity on this medium. However, clones with higher and lower activity than the parent strain were observed at about 1% frequency. Two clones each with nitrogenase activity lower (S24A06, S24EB12; 90% less) and higher

(S24A044, S24A050; 30% more) than the parent strain S24, were then selected for detailed cultural and symbiotic studies. Table 1 shows the nitrogenase activity of these strains in media containing various sugars, citric acid cycle intermediates and amino acids. The two mutants S24A06 and S24EB12 which showed low activity on yeast extract-mannitol-glutamate-succinate-agar medium, also showed comparatively low activity on all the media tested. Contrary to this, the mutant S24A044 and S24A050 showed constantly higher activity.

Table 1. Nitrogenase activity of *Vigna* rhizobium S24 and its mutants under culture conditions

Medium	nmol C_2H_2 reduced/h/mg cell protein				
	S24	S24EB12	S24A06	S24A044	S24A050
YEMGA[a]	3.2	4.0	3.4	4.2	4.2
a +Fumarate	82.9	6.8	8.2	116.7	124.2
a +Malate	114.2	8.2	8.4	134.2	127.4
a +Succinate	72.6	5.4	6.2	89.2	95.2
a +Pyruvate	60.3	8.4	8.6	79.2	75.2
a +Glutarate	56.5	6.2	6.4	74.5	73.2
Sugar substitutes[b]					
Galactose	120.6	5.4	6.0	142.2	128.4
Mannose	151.0	10.2	12.5	182.4	170.2
Glucose	89.4	6.5	6.4	104.4	110.3
Amino acid substitutes[c]					
Aspartate	78.4	6.2	5.4	114.2	120.2
Proline	112.2	5.2	5.2	150.2	135.4
Lysine	86.2	5.2	5.4	112.4	104.2
Serine	72.0	6.2	5.4	96.4	98.4
Glutamine	89.2	8.2	8.4	104.6	115.4
Asparagine	90.4	8.4	8.8	116.4	104.2

The citric acid cycle intermediates, sugars and amino acids were added in the YEMGA medium at 10, 55 and 2 mM concentrations, respectively. No activity was detected in media containing citrate, arabinose, xylose and ribose. C_2H_2 was injected after 10 days of growth in tubes (air: C_2H_2, 9:1). The assay tubes were further incubated for 24 h and C_2H_2 formed was analysed.

[a] Yeast extract-mannitol-glutamate-agar medium.

[b] To yeast extract-glutamate-fumarate agar medium, the sugars indicated were added.

[c] To yeast extract-glucose the amino acids indicated were added.

In media containing different levels of succinate (0 to 60 mM) the mutan S24A06 and S24EB12 showed lower activity, whereas, S24A044 and S24A05 showed high activity as compared with S24 at all the concentrations of succinat (table 2).

Table 2. Effect of succinate levels on nitrogenase activity of *Vigna* rhizobium S24 and i mutants under culture conditions.

Succinate (mM)	nmol C_2H_2 reduced/h/mg cell protein				
	S24	S24EB12	S24A06	S24A044	S24A050
0	3.5	4.0	3.9	5.2	4.4
5	45.4	6.9	7.4	52.4	39.7
10	86.2	12.1	7.1	112.4	104.2
20	95.4	14.4	13.4	123.8	115.7
40	84.2	16.6	13.2	116.4	114.8
60	74.2	14.9	12.9	98.2	79.2

Succinate was added to yeast extract-manitol-glutamate-agar medium.

Table 3 shows a comparison between the mutants and the parent, of nodule nitrogenase activity, nodule weight, plant weight and plant nitrogen ratios at different stages of growth. In all the strains, except at 15 days of plant growth, the nodule weight per plant was comparable with the parent strain indicating no change in host infectivity. The two mutants S24A06 and S24EB12, with low nitrogenase activity under cultural conditions, showed nodule nitrogenase activity as well as plant weight and nitrogen ratios lower than the parent strain. This might mean that the nitrogenase activity in the culture medium has a close relationship with the *in vivo* nitrogen-fixing capacity of the strains. However, nodule nitrogenase activity, plant weight and plant nitrogen content of strains having increased nitrogenase activity in culture medium, were comparable with those with the parent strain S24. This shows that perhaps the plant system does not allow the full expression of the higher nitrogen-fixing capacity of these bacteria inside nodules.

The parent strain S24 has also a hydrogen-oxidizing hydrogenase system (Dadarwal *et al.*, 1980). Emerich *et al.* (1979) observed that nitrogenase activity in a bacteroid suspension of *R. japonicum* having such a hydrogenase system, is increased substantially in 10% H_2 atm. This increase in C_2H_2 reduction rates per unit weight of nodules in a hydrogen atmosphere could be taken as a measure of the hydrogenase system. When detached nodules of plants inoculated with the different strains were incubated in a 10% H_2 atm, enhanced rates of C_2H_2 reduction were observed in all the cases (table 4). This showed that the hydrogenase systems in nodules of the mutants were unaltered and that the low nitrogen fixation in the two mutants with lower activity was not due to the lack of an electron recycling system.

Table 3. Comparative effectivity of *Vigna* rhizobium S24 and its mutants on green gram var. K851.

Plant age (days)	Observation	Strains tested				
		S24	S24EB12	S24A06	S24A044	S24A050
15	Nitrogenase activity [a]	30.7	6.4	11.9	30.5	29.3
	Nodule weight [b]	6	6	10	7	12
	Shoot weight ratio [c]	1.74	1.11	1.58	1.74	2.21
	Shoot N ratio [d]	1.92	1.20	1.49	1.87	1.98
25	Nitrogenase activity	167.2	37.7	10.3	180.7	189.2
	Nodule weight	36	33	32	39	29
	Shoot weight ratio	2.39	1.63	1.47	2.50	2.45
	Shoot N ratio	2.41	1.54	1.52	2.46	2.44
35	Nitrogenase activity	211.6	35.7	28.8	179.7	182.8
	Nodule weight	63	68	57	56	60
	Shoot weight ratio	3.68	2.02	2.05	3.60	3.80
	Shoot N ratio	3.82	2.21	2.12	3.71	3.81

[a] Nitrogenase activity of intact nodules expressed as μmol C_2H_2 reduced/h/g dry weight of nodules.

[b] Nodule weight is expressed as mg/plant.

[c] $\frac{\text{Plant dry weight in inoculated}}{\text{Plant dry weight in control}}$

[d] $\frac{\text{Plant nitrogen in inoculated}}{\text{Plant nitrogen in control}}$

The ratios are calculated from average values of 15 plants.

Table 4. Effect of added hydrogen on nitrogenase activity of detached nodules of mung bean inoculated with different strains after 25 days of plant growth.

Rhizobium strains	μmol C_2H_2 reduced/g fresh nodule weight			
	without hydrogen		with hydrogen	
	4 h	24 h	4 h	24 h
S24	4.04	5.09	6.87	7.45
S24EB12	2.74	3.74	3.66	5.07
S24A06	1.41	2.49	1.91	3.41
S24A044	4.07	5.08	6.24	7.41
S24A050	4.66	4.86	6.24	8.33

The values are the cumulative values at 4 and 24 h after injecting the C_2H_2. The nodules were incubated in air: C_2H_2 (9:1) and air: C_2H_2 : H_2 (8:1:1) atmosphere without and with H_2, respectively.

Discussion

A limited number of slow growing strains of *R. japonicum* and *Rhizobium* sp. 'cow pea group' are known to express nitrogenase activity under culture conditions (Child and Kurz, 1978; Wilcockson and Werner, 1978; Kennedy and Pankhurst, 1979). However, none of these workers have correlated the N_2-fixing ability of the strains with the nitrogenase activity under the free living conditions. One of the reasons for this is that all the *Rhizobium* strains infecting a particular legume do not express nitrogenase activity under culture conditions in the same medium.

In the present study mutants with decreased and increased nitrogenase activity were derived from a N_2 fixing strain. These mutants were not defective in nodulation. Strains S24A06 and S24EB12 with decreased nitrogenase activity under free living conditions were less effective than the parent strain in nitrogen fixation (table 3). However, contrary to expectations, the mutants S24A044 and S24A050 with increased nitrogenase activity did not show increased nitrogen fixation in the host as compared with the parent strain, although studies with different levels of succinate in the culture medium showed that both the strains had a higher specific activity of nitrogenase than the parent (table 2). The limitations, therefore, could exist in the host system where the nitrogenase potential of the rhizobial strain is not expressed beyond a certain limit. However, this does not rule out the possibility of better effectiveness of these strains in other genotypes of the same host, which have not been tested.

The nitrogenase-dependent H_2 evolution is one of the major reasons for low effectivity of rhizobia in legume symbiosis (Albrecht *et al.,* 1979). The parent strain S24 as well as the four mutants possess the H_2 recycling system (table 4). Therefore, the low effectivity of the mutants S24A06 and S24EB12 was not due to a change in this enzyme system. This is the first report that correlates symbiotic effectivity with nitrogenase activity under culture conditions, showing limitations offered by the plant system to the expression of nitrogenase in nodules. However, it is possible that *in vitro* nitrogenase activity may not be an indicator of *in vivo* performance. Similar studies need to be carried out with other strains known to express nitrogenase in free living conditions.

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Localization of glucose-6-phosphatase and fructose-1-6-diphosphatase in subcellular fractions from different regions of the rat brain

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Abstract. Two key enzymes of gluconeogenesis, glucose-6-phosphatase and fructose-1-6-diphosphatase, were present in the cerebral hemispheres, the cerebellum and the brain stem of the rat brain. Significant activities of these enzymes were associated with the particulate fraction.

Keywords. Glucose-6-phosphatase; fructose-1-6-diphosphatase; brain; subcellular localization.

Introduction

Gluconeogenesis comprises the synthesis of glucose from non-carbohydrate sources like lactate, pyruvate, glycerol and amino acids. The activity, regulation and general properties of gluconeogenic enzymes have been well studied in the liver and the kidney (Exton, 1972). However, the existence and activity of gluconeogenic enzymes has not been adequately studied in the brain. The presence of these enzymes in the brain has attracted the attention of investigators only recently (Cheng and Cheng, 1972; Patel, 1974). In the present study, an attempt has been made to localize two gluconeogenic enzymes, glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.7) and fructose-1-6-diphosphatase (fructose-1-6-diphosphate I phosphohydrolase, E.C. 3.1.3.11) in three regions of the rat brain, namely, cerebral hemispheres, the cerebellum and the brain stem.

Glucose-6-phosphatase occupies an unique metabolic position, catalyzing the terminal reaction not only in gluconeogenesis but also in glycogenolysis (Ashmore and Weber, 1959). Its presence in chicken brain has recently been reported by Richardson *et al.* (1979). Fructose diphosphatase catalyzes one of the irreversible steps in gluconeogenesis and serves as a site for the regulation of this process (Tejwani *et al.*, 1976).

Materials and methods

Adult rats of Holtzman strain weighing between 180-220 g were used. The animals were killed by cervical dislocation and the brains dissected out immediately. Each brain was divided into three regions, cerebral hemisphere, cerebellum and brain stem. Tissues were homogenized (1:10) using a Potter Elvehjem-type homogenizer fitted with a teflon plunger in 0.25 M sucrose-buffered with 20 mM triethanolamine (pH 7.4) containing 0.1 mM dithiothreitol. Supernatant and crude mitochondrial fractions were prepared by differential centrifugation according to the method o Whittaker (1959). The mitochondrial fraction was washed once with the homogenizing medium before assaying for enzyme activity.

Glucose-6-phosphatase was estimated according to the method of Baginski *et al* (1974) with some modifications. The reaction mixture contained the following components in a final volume of 0.4 ml; Tris-HCl bufer, 135 mM (pH 6.5); EDTA, 5 mM and glucose-6-phosphatase, 20 mM. The reaction was initiated by the addition of enzyme extract (100 µl in the case of brain region and 50 µl in the case of liver and kidney were used per assay) and incubated for 15 min at 30°C. The reaction was stopped by adding 1 ml of 10% ice-cold trichloroacetic acid. The precipitate was centrifuged (800 *g*) and 0.3 ml of the clear supernatant was used for the estimation of inorganic phosphate (P_i) by the method of Chen *et al.* (1956) Absorbance was recorded at 820 nm in a Carl Zeiss Spectrophotometer.

Fructose diphosphatase activity was determined in the different regions of the brain according to the method of Toshima and Yoshimura (1975) with some modification. The reaction mixture contained the following components in a final volume of 0.4 ml: Tris-HCl buffer, 135 mM (pH 7.5); $MgCl_2$, 14 mM and fructose diphosphate, 5 mM. The reaction mixtures were assayed at 30°C for 15 min and P liberated was estimated as described for the assay of glucose-6-phosphatase.

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. All chemicals were of Analar grade. Substrates were obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

Results and discussion

The activities of glucose-6-phosphatase and fructose diphosphatase (mitochondrial and soluble) in different regions of the rat brain are presented in table 1, along with protein values. The activities of the two enzymes were also assayed in liver and kidney.

The results obtained in the present study showed that high percentages of these two gluconeogenic enzymes were present in the mitochondrial fraction (glucose-6-phosphatase was 52.4%, 47.6% and 48.5% and fructose diphosphatase 47.7%, 31% and 30.2% in the cerebral hemisphere, the cerebellum and the brain stem respectively, normalizing the activity in the supernatant+mitochondrial fraction to 100%). Recently it was shown that glucose-6-phosphatase is present in hepatic mitochondrial preparations of some vertebrates (Vorhaben and Campbell, 1979). It is well known that the amount of gluconeogenesis in the adult brain is very low (Balazs, 1970) as compared to liver and kidney. The other two gluconeogenic enzymes, pyruvate carboxylase (EC 6.4.1.1) and phosphenol pyruvate carboxykinase (EC 4.1.1.32) have already been shown to be present in brain (Cheng and Cheng, 1972; and Patel, 1974) and both these enzymes in brain have been shown to be associated with the mitochondrial fraction. The present data also shows

Table 1. Glucose-6-phosphatase and fructose diphosphatase activites in different regions of rat brain.

	Glucose-6-phosphatase nmol/g tissue/min		Fructose diphosphatase nmol/g tissue/min		Protein mg/g tissue	
	A	B	A	B	A	B
Cerebral hemispheres	186±20	203± 7	350±40	320±80	56± 5.1	84.3±23
Cerebellum	220±72	200± 9	400±60	180±50	48± 7.1	63.8±9.9
Brain stem	220±60	190±70	370±85	160±70	56.3±13.8	76.8±7.2

A = Supernatant fractions; B = Mitochondrial fraction.

Each value is a mean ± SEM of more than four values. The activity of glucose-6-phosphatase in liver and kidney is 6.04 ±1.24 and 7.1 ±2.02 and fructose diphosphatase, 2.11 ±0.47 and 5.0 ±0.87 µmol/g/min respectively.

that glucose-6-phosphatase and fructose diphosphatase which control the rate of gluconeogenesis are present in significant amounts in the mitochondria. Also, some key glycolytic enzymes like hexokinase, pyruvate kinase and aldolase are localized in the particulate fraction of the brain (MacDonnell and Greengard, 1974).

The presence of all the gluconeogenic enzymes in the brain indicates that the complete pathway is operative and may be of significance under conditions where enough intracellular glucose is not available for utilization, although the circulating blood glucose levels may be high. One such hormonal condition is diabetes where the absence of insulin adversely influences the normal transport of glucose into the cells. The availability of these enzymes may also be beneficial to the brain under conditions like hypoglycemia that may be caused by drugs.

Preliminary data from our laboratory (to be published elsewhere) show a differential effect of alloxan diabetes on the activities of the two enzymes in the three regions of the rat brain at different time intervals after the onset of diabetes. Administration of insulin to diabetic animals showed a differential effect in all the three regions. Based on these observations it can be postulated that glucose-6-phosphatase and fructose diphosphatase may have a functional role in carbohydrate metabolism in the brain.

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Toluene toxicity: Effects of sublethal levels on enzyme activities in seawater adapted tilapia (*Sarotherodon mossambicus* Peters)

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Abstract. The effects of exposing seawater adapted cichlid fish, tilapia (*Sarotherodon mossambicus* Peters) to sublethal concentrations of toluene on the activities of lactate dehydrogenase, succinate dehydrogenase and acetylcholinesterase were studied. The activity of lactate dehydrogenase increased while those of succinate dehydrogenase and acetylcholinesterase were inhibited in most tissues of the exposed fish. The alterations in the dehydrogenases suggested that some changes in carbohydrate metabolism may have occurred. Acetylcholinesterase inhibition in brain and other tissues indicated impairment of nervous function in toluene-intoxicated tilapia.

Keywords. Tilapia; toluene toxicity; lactate dehydrogenase; succinate dehydrogenase, acetylcholinesterase.

Introduction

A large number of studies on the biological effects of oil pollution in the aquatic environment deal with the effects of whole crude or refined oils or their water-soluble fractions. However, low boiling, aromatic hydrocarbons which are probably the most toxic constituents of oil have until now not been examined in sufficient detail. Toluene, benzene and naphthalene constitute a major component of various oils (Anderson *et al.*, 1974). They may be readily lost by weathering but are toxic in waters that are relatively stagnant and are chronically polluted. Korn *et al.* (1977) have stated that toluene is more toxic than many other hydrocarbons such as benzene, though the latter are more water-soluble. Toxicity of toluene to fish has been demonstrated (Pickering and Henderson, 1966; Stoss and Haines, 1979).

The present study forms part of a detailed programme for investigating the metabolic impact of some important petroleum hydrocarbons on aquatic animals. In this paper, the effects of sublethal concentrations of toluene on the activities of lactate dehydrogenase (EC 1.1.1.27), succinate dehydrogenase (EC 1.3.99.1) and acetylcholinesterase (EC 3.1.1.7) in different tissues of the cichlid fish tilapia (*Sarotherodon mossambicus* Peters) adapted to seawater are reported. Tilapia, a euryhaline fish, easily adapts to saline waters and rapidly recovers from the initial osmotic shock (Meenakshi *et al.*, 1979). However, the fish adapted to 100%

seawater showed high mortality rates (65-85%) during the first four weeks of exposure to toluene. Hence, the effects of toluene on fish adapted to 50% seawater only are reported here.

Materials and methods

Tilapia were collected from Shivaji lake, Thane, Maharashtra and maintained in the laboratory in large glass aquaria containing weathered, well-aerated tap water for two weeks. They were then transferred to aquaria containing 10%, 20%, 30%, 40% and 50% seawater. An acclimation period of one week was allowed at each transfer, but in 50% seawater, the fish were maintained for four weeks. The seawater acclimated fish did not show any visible signs of stress and were seen to swim about actively. During the entire acclimation period, the fish were fed daily with live tubificid worms (*Tubifex* sp.). The fish were also supplied with groundnut cake once a week.

Since enzyme activities in tilapia are related to size (Mansuri and Pandya, 1977), fish of a relatively uniform size (12-15 cm long; 22.7-24.1 g) were selected from the stock for the exposure. They were further acclimated in the experimental aquaria for two days and then exposed to toluene (25 or 50 mg/L) for ten weeks. The level of toluene was kept constant by changing the water everyday and adding the requisite amounts of a toluene stock solution (prepared in Analar acetone to get more accurate concentrations). The fish were fed daily, prior to change of water to prevent ingestion of toluene through food. A control group exposed to acetone alone was also maintained.

Water samples from each aquarium were analyzed once a week. The conditions prescribed by the American Public Health Association, American Water Works Association and Water Pollution Control Federation (1971) for the quality of water during static exposure of aquatic animals to pollutants were maintained throughout the experiment. The values of some of the physicochemical characteristics of the water from the control aquaria were: dissolved oxygen—7.3 mg/L; dissolved chlorine—0.018 mg/L. salinity—16.8 parts per thousand; pH 7.7; conductivity—18.5 mmhos/cm; temperature—27.5°C. There was no noticeable change in the quality of the water upon addition of toluene.

At the end of ten weeks, the fish were killed by decapitation and the tissues rapidly excised. The tissues were rinsed, blotted and homogenzied in a motor-driven, all glass homogenizer with two volumes of chilled saline (0.7% NaCl). Homogenates were centrifuged (Model 101, MB Corporation, Bombay) at 10,000 g for 15 min. The supernatant fractions were diluted with ten volumes of chilled saline and used as the enzyme source. The activities of lactate dehydrogenase, succinate dehydrogenase and acetylcholinesterase were determined according to the methods described by Srikantan and Krishnamurthy (1955), Nachlas *et al.* (1960) and Pilz (1965), respectively. Protein content was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

The difference in enzyme activities between the control and the exposed fish were analyzed for statistical significance according to the Student's 't'-test (Spiegel, 1961).

Results

The stress of exposure to toluene stimulated lactate dehydrogenase activity in all the tissues (table 1). The increase in activity was most significant in liver and brain. The enzyme activity was not markedly changed in the kidney and the gonads. On the other hand, succinate dehydrogenase activity decreased in all the tissues of the exposed fish (table 2), the most marked reduction being in liver and brain.

Table 1. Effect of toluene exposure on lactate dehydrogenase activity in tilapia.

Tissue	Control	Expt. 1 (25 mg/litre)	Expt. 2 (50 mg/litre)
Liver	74 ± 7	121 ± 6^a (+64%)	252 ±21^a (+241%)
Brain	67 ± 5	126 ± 6^a (+88%)	186 ±15^a (+178%)
Cardiac muscle	59 ± 4	89 ± 5^b (+51%)	107 ± 8^b (81%)
Skeletal muscle	32 ± 2	53 ± 2^b (+66%)	82 ± 4^b (+156%)
Gill	17 ± 3	22 ± 4 (+29%)	24 ± 4^b (+41%)
Intestine	89 ± 9	129 ±11^a (+45%)	159 ±17^a (+79%)
Kidney	142 ±12	175 ±14^b (+23%)	189 ±12^a (+33%)
Testes (immature)	23± 2	28 ± 4 (+22%)	32± 4^b (+39%)
Ovary (immature)	29 ± 2	34 ± 4 (+17%)	36 ± 5^b (+24%)

^{a}–$P<0.001$; ^{b}–$P<0.01$.

Sp. activity—µmol of acetylecholine hydrolyzed/mg protein/h. Values are mean ±SD of 7 and 6 determinations in the control and exposed fish respectively. Values in the paranthesis represent change over control.

Table 2. Succinate dehydrogenase activity in tilapia following toluene exposure.

Tissue	Control	Expt. 1 (25 mg/litre)	Expt. 2 (50 mg/litre)
Liver	187 ±10	101 ±15^a (−46%)	58 ±14^a (−69%)
Brain	376 ±29	163 ±13^a (−57%)	129 ±17^a (−66%)
Cardiac muscle	68 ± 8	47 ±12^b (−31%)	36 ± 6^a (−47%)
Skeletal muscle	62 ± 6	34 ± 5^a (−45%)	22 ± 8^a (−65%)
Gill	76 ± 9	62 ± 9 (−13%)	48 ± 7^a (−36%)
Intestine	205 ±21	172 ±20 (−16%)	135 ±16^a (−34%)
Kidney	192 ± 8	164 ±19^b (−15%)	132 ±16^a (−31%)
Testes (immature)	141 ±13	116 ±12^b (−18%)	81 ± 9^a (−43%)
Ovary (immature)	109 ± 7	72 ± 7^a (−34%)	62 ± 9^a (−43%)

^{a} – $P < 0.001$; ^{b} – $P < 0.01$.

(Sp. activity—nmol of formazan formed/mg protein/h. Values are mean ±SD of 7 and 6 determinations in the control and exposed fish respectively. Values in the parenthesis represent change over control).

Acetylcholinesterase activity was significantly inhibited in most tissues of tilap following exposure to toluene (table 3). Tissues like brain, skeletal and cardi muscles showed the highest inhibition in the enzyme activity.

Table 3. Acetylcholinesterase activity in control and toluene treated tilapia.

Tissue	Control	Expt. 1 (25 mg/litre)	Expt. 2 (50 mg/litre)
Liver	31.82 ±2.17	26.42 ±1.87^b (−17%)	22.38 ±1.90^a (−30%)
Brain	81.97 ±5.03	59.98 ±3.19^a (−27%)	27.54 ±4.32^a (−66%)
Cardiac muscle	65.21 ±3.19	51.03 ±2.19^a (−22%)	34.55 ±2.06^a (−47%)
Skeletal muscle	56.01 ±3.77	44.88 ±4.35^b (−20%)	31.14 ±3.43^a (−44%)
Gill	46.72 ±3.63	38.26 ±2.49^b (−18%)	27.52 ±3.02^a (−41%)
Intestine	34.25 ±3.08	29.11 ±2.03^b (−15%)	23.52 ±1.94^a (−31%)
Kidney	18.43 ±1.04	16.63 ±1.11 (−13%)	14.74 ±0.07^a (−20%)
Testes (immature)	32.93 ±2.30	27.02 ±1.51^b (−18%)	22.07 ±1.47^a (−33%)
Ovary (immature)	24.47 ±2.34	21.29 ±1.21 (−15%)	18.86 ±0.86^a (−23%)

$a - P < 0.001$; $b - P < 0.01$.

Sp. activity µmol of acetylecholine hydrolyzed/mg protein/h. Values are mean ±SD of 7 an determinations in the control and exposed fish respectively. Values in the paranthe represent change over control.

Discussion

Stimulation of lactate dehydrogenase, an enzyme associated with the anaero pathway of carbohydrate metabolism and simultaneous inhibition of succinate hydrogenase, one of the important enzymes involved in the Kreb's cycle, in t tissues of the toluene-intoxicated tilapia indicate disturbances in the cellu oxidative processes. The changes appear to favour a less efficient anaero metabolism in tilapia in response to the environmental stess, probably due to t inability of the tissues of such fish to derive sufficient oxygen for the nor metabolic functions. Such tissue hypoxia was indicated by an increase in lactic acid/pyruvic acid ratio, accompanied by an increase in lactate dehyd genase and decrease in succinate dehydrogenase activities, in tissues of tila exposed to lethal and sublethal levels of naphthalene (Dange, 1979).

In the natural environment, oil pollution may lead to a drastic reduction in oxygen tension of water, especially in the stagnant, confined types of waters. T possibility of the dissolved hydrocarbon affecting the tissue enzymes by decreas dissolved oxygen content of the water may be ruled out while interpreting d from the present study, since it was observed that toluene did not reduce oxygen content significantly. The suggested lack of a proper oxygen supply to tissues may be due to some damage to the gills similar to that observed in f exposed to different petroleum hydrocarbons (Gardner, 1975; DiMichele a Taylor, 1978). Gill hyperplasia was also seen in tilapia treated with naphthal and toluene (Dange, unpublished data). This is consistent with Hodson's (1976) view that aquatic pollutants cause gill damage leading to tissue hypoxia and possibly death.

Moreover, aromatic hydrocarbons like toluene, being lipid-soluble, may affect the erythrocyte membrane (Gerarde, 1960). Berry (1980) suggested that due to this, the supply of oxygen to various tissues would be inadequate to meet the increased metabolic demands of the stressed fish. Under such conditions, fish like tilapia which are capable of anaerobic metabolism may be able to produce the necessary amount of energy by increasing lactate dehydrogenase activity, probably by utilising stored glycogen as in the case of tilapia exposed to naphthalene (Dange, 1979).

Levitan and Taylor (1979) showed that the effects of petroleum hydrocarbons on fish are salinity-dependent, due to the increased energy demands on an already stressed osmoregulatory system of the euryhaline fish. Probably the metabolic burden of pollution is aggravated by the stress due to the hypertonic medium. However, preliminary studies had shown that no significant effects on the enzyme activity could be ascribed to osmotic stress in the seawater adapted tilapia.

The response of tilapia to toluene included the inhibition of acetylcholinesterase in all the tissues, indicating a disruption in the nervous system in the exposed fish. As this enzyme is involved in the maintenance of the structural and functional integrity of cellular membranes (Kutty *et al.*, 1976), it may be inferred that toluene causes disturbances in the normal cellular functions. Although a large decrease in the acetylcholinesterase activity was not observed by us, as observed by Coppage (1972), the decrease was larger than the 10% decrease suggested as an acceptable criterion for assessing water quality (Nicholson, 1967). Thus even the lower sublethal concentration of toluene (25 mg/L) would be objectionable.

The effects on these enzymes probably are only a part of the general metabolic response to stressful conditions and may not be specific for toluene. Hydrocarbons such as benzene and toluene are irritants of mucus membranes and the changes in enzymes like the dehydrogenases may be induced secondarily as a result of lesions in gills and other tissues. The effects may also be caused by accumulation of toluene in different tissues. The generally more severe effects on enzyme activities in liver may be due to extensive accumulation and metabolism of these hydrocarbons in this organ.

Changes in the lactate dehydrogenase and succinate dehydrogenase activity may indicate the facility with which tilapia can shift to anaerobic metabolism under adverse conditions and it would be interesting to observe if similar enzymatic changes occur more often in the anaerobic type of fish following exposure to pollutants.

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Spectrophotometric assay of immobilized tannase

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Abstract. A procedure for the assay of immobilized tannase with polyacrylamide gel, collagen and Duolite-S-762 as matrices is described. It is based on the spectrophotometric determination of gallic acid formed by the enzymatic hydrolysis of tannic acid. The kinetic parameters of the enzymatic reaction have been studied and an assay procedure has been formulated. This method appears to be much more accurate than those reported earlier.

Keywords. Tannase; immobilization; gallic acid; tannic acid; spectrophotometric assay.

Introduction

Tannase (Tannin acyl hydrolase EC.3.1.1.20) is an enzyme which catalyses the hydrolysis of tannic acid (Weetall and Detar, 1974). It also acts on esters of gallic acid commonly found in tea extracts. The hydrolysis of these esters enhances the solubility of tea in cold water (Coggon *et al.*, 1975). Hence soluble tannase has been recommended for use in the preparation of instant tea (Sanderson and Coggon, 1977). Use of immobilized tannase may have advantages in the preparation of instant tea. While the assay of soluble tannase is simple (Iibuchi *et al.*, 1967; Coggon *et al.*, 1975), that of the matrix bound tannase is more complicated. A method described by Weetall and Deter (1974) for the assay of immobilized tannase is not satisfactory because of high error and does not appear to be easy. Therefore, there is a need for an easy and reliable method of assay. The method described below is based on the determination of gallic acid formed from the tannic acid instead of measuring the disappearance of tannic acid, which is the basis of the method of Weetall and Detar (1974).

Materials and methods

Tannic acid was obtained from Bush Co. Ltd., London, UK, and purified according to the procedure of Iibuchi *et al.* (1967). Gallic acid was obtained from the British Drug House, Poole, England and recrystallized from alcohol. Tannase and collagen hydrochloride were gifts from Enzyme Development Corporation, New York, USA, and FMC Corporation, Nutley, New Jersey, USA respectively. Glutaraldehyde was a product of E. Merck, Darmstadt, Germany. Duolite-S-762 was purchased from Diamond Shamrock Corporation, Cleveland, Ohio, USA.

Acrylamide and N-N′-methylene bis-acrylamide were obtained from Koch-Light Laboratories, Colnbrook, England. All other chemicals used were of analytical grade.

Tannase (soluble) had about 1200-1500 units of activity per mg enzyme; one unit is equivalent to that amount of enzyme which causes a change in absorbance of 0.01 at 250 nm per min at 25°C. When assayed at 310 nm (disappearance of tannic acid, 0.01 absorbancy change/min at 25°C is equivalent to one unit) using 0.008% tannic acid as substrate, the enzyme had a specific activity of 1200-1800.

Enzyme immobilization

(i) *Entrapment in polyacrylamide gel:* Tannase (100 mg) was dissolved in 20 ml of 50 mM sodium acetate buffer (pH 4.7) and entrapped in polyacrylamide gel according to the procedure of Chibata *et al.* (1976). The weight of the polyacrylamide gel containing tannase was about 26 g (drained weight). The washings contained 28% and the gel retained about 72% of the original activity.

(ii) *Complexing with collagen:* Tannase (100 mg) was mixed with 50 ml of 1% collagen hydrochloride in 50 mM sodium acetate buffer (pH 4.7) and cross-linked with glutaraldehyde according to the procedure of Vieth *et al.* (1973). The dry weight of the enzyme-bound collagen membrane was about 620 mg with about 90% of the original activity.

(iii) *Tannase on Duolite-S-762:* Tannase (100 mg) in 10 ml of 50 mM sodium acetate buffer (pH 4.7) was adsorbed onto 10 g Duolite-S-762 and cross-linked with glutaraldehyde according to the procedure of Olson and Stanley (1973). Drained weight of enzyme-bound Duolite-S-762 was about 12.5 g and washings collected from it had about 22% of the original activity: Duolite-S-762 retained about 78% of the original activity.

(iv) *Tannase co-valently attached to cynuric chloride activated cellulose:* An amino chloro-S-triazinyl derivative of cellulose was prepared by reacting cellulose (20 g) with 2-amino-4,6, dichloro-S-triazine (2 g, prepared from cynuric chloride. Tannase (200 mg in 20 ml of 0.05 M sodium acetate buffer, pH 4.7) was co-valently attached to the amino chloro-S-triazinyl derivative of cellulose according to the procedure of Lilly (1976). This immobilized enzyme preparation was found to be very active intially but lost its activity within 50 hours.

Enzyme assays

(i) *Assay for soluble tannase:* The assay procedure for soluble tannase was modified to suit our experimental conditions and to compare its activity with that of immobilized tannase. In this assay procedure, the product (gallic acid) was determined at 250 nm instead of measuring the disappearance of tannic acid at 310 nm.

The enzyme (2-5 µg/ml) and the substrate (0.008%) were prepared in 50 mM sodium acetate buffer (pH 4.7). The enzyme and substrate were replaced by buffer in both control (without enzyme) and blank (without enzyme and substrate). The incubation mixtures in a total volume of 10 ml containing 9.8 ml substrate and

0.2 ml enzyme were incubated for 20 min at 25°C. The reaction was stopped b heating the tubes in a boiling water bath. After cooling them to room temperatur (25°C),the unhydrolyzed tannic acid was removed by adsorption on polyacrylamid gel (1 g), by shaking in a gyratary shaker for 1 h. The reaction mixtures wer filtered and the filtrates read at 250 nm against a blank, in a Beckman spectro photometer (Model 26). The absorption at 250 nm for control was negligible, an the difference in absorbance at 250 nm between test and control was taken as measure of soluble tannase activity.

(ii) *Immobilized tannase assay:* The assay of tannase entrapped in polyacryla mide gel is described in the following procedure. Ten ml of 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7) was added separately to the enzyme-free and enzyme-entrapped polyacrylamide gel (1 g each) and incubated in a gyratary shaker (120 rpm) for 20 min at 25°C. The absorbance of the filtrates wa determined at 250 nm against a blank of 50 mM sodium acetate buffer (pH 4.7). The difference in absorbance (Δ A_{250}) obtained between the reaction mixtures containing the enzyme and that without enzyme was essentially due to gallic acid released by the action of the enzyme and was taken as a measure of immobilized tannase activity. A unit of activity is defined as the amount of enzyme which causes a change in absorbance at 250 nm of 0.01 per min at 25°C. Other systems such as tannase immobilized on collagen membrane and Duolite-S-762 were also assayed similarly.

Results and Discussion

The absorption spectra of tannic and gallic acids, the substrate and the product of the reaction catalysed by tannase are shown in figure 1. The ϵ_m (molar extinction coefficient) of tannic acid (molecular weight 1700-1800) and gallic acid (molecular weight, 170) are 27,200 and 4910 respectively at 250 nm and 32,400 and 555

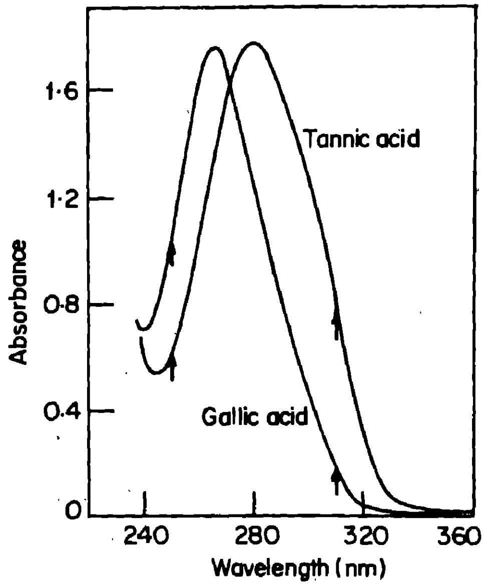

Figure 1. Absorption spectrum of tannic acid (40 µg/ml) and gallic acid (40 µg/ml) in 50 mM sodium acetate buffer (pH 4.7).

respectively at 310 nm. Hence either the disappearance of tannic acid or the formation of gallic acid can be used as a measure of tannase activity by measuring the changes in absorbance at 310 nm (for tannic acid) or at 250 nm (for gallic acid). Assay of soluble tannase by measuring the disappearance of tannic acid at 310 nm is quite satisfactory (Iibuchi *et al.*, 1967; Coggon *et al.*, 1975). Soluble tannase has also been assayed using methylgallate as substrate (Yamada *et al.*, 1968). However these methods have not been found suitable for the assay of matrix-bound tannase due to small difference in UV absorption of methylgallate and gallic acid, and absorption of tannic acid.

Tannic acid is absorbed by many compounds particularly charged molecules such as proteins, polyamines, etc. (Hurst and Turner, 1962; Haurowitz, 1950). Adsorption of tannic acid during the assay, either by soluble compounds or by matrices used for binding can lead to erratic results, when tannic acid disappearance is used as a measure of enzyme activity. This may have been one of the reasons for the rather large errors in the assay procedures of Weetal and Detar (1974). Different matrices with and without tannase were tested with a view to determine whether they adsorbed tannic acid, and if so, to what extent. The results show that tannic acid is adsorbed by the matrices used, and even after 30 min incubation, the absorbance at 310 nm is almost minimal and adsorption of tannic acid occurs to nearly 90% (table 1). In the presence of the enzyme, the high initial absorbance at 250 nm due to tannic acid, decreases at first presumably due to adsorption of tannic acid, and then absorption at 250 nm increases apparently due to release of gallic acid.

Table 1. Hydrolysis and adsorption of tannic acid on matrices with and without tannase.

Period of contact	Enzyme-free matrices						Enzyme-immobilized matrices					
	Poly-acrylamide gel[a]		Duolite S-762[b]		Collagen		Poly-acrylamide gel[a]		Duolite-S-762[b]		Collagen	
(min)	Absorbance (nm)						Absorbance (nm)					
	250	310	250	310	250	310	250	310	250	310	250	310
0	0.94	1.05	1.19	1.29	1.22	1.32	1.15	1.14	1.55	0.62	1.61	1.60
10	0.40	0.32	0.28	0.16	0.57	0.51	0.60	0.36	0.78	0.16	1.41	0.77
20	0.20	0.19	0.28	0.13	0.42	0.42	0.76	0.28	0.92	0.16	1.66	0.41
30	0.19	0.15	0.25	0.10	0.34	0.30	1.16	0.28	0.98	0.17	1.64	0.35
60	0.18	0.15	0.25	0.10	0.17	0.11	1.29	0.26	1.17	0.12	1.84	0.24

The reaction mixtures were incubated in a gyratary shaker (120 rpm) at 25°C for various time periods. Then they were filtered and absorbance of the filtrates were measured at 250 and 310 nm.

[a] One g polyacrylamide gel (enzyme-free and enzyme-entrapped) + 10 ml 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7).

[b] One g Duolite-S-762 (enzyme-free and enzyme-immobilized) + 10 ml 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7).

[c] One hundred mg collagen (enzyme-free and enzyme-complexed) + 10 ml 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7).

In view of the above results, experiments were conducted to ascertain whether gallic acid is also adsorbed by the matrices. The results (table 2) indicate that gallic acid recovery is almost 100% unlike in the case of tannic acid (table 1) at all the concentrations tested. It is therefore quite clear that gallic acid is not adsorbed by the polyacrylamide gel. Similar results were obtained with Duolite-S-762 and collagen membrane. Therefore the release of gallic acid by the enzymatic hydrolysis of tannic acid can be used as a satisfactory measure of immobilized tannase activity.

Table 2. Behaviour of gallic acid on polyacrylamide gel

Concentration of gallic acid in 50 mM sodium acetate buffer (pH 4.7) %	Absorbance of filtrates at 250 nm			
	Control[a]	Gallic acid	Gallic acid with enzyme entrapped polyacrylamide gel	Gallic acid with enzyme free polyacrylamide gel
0.002	0.56	0.61	0.60	0.61
0.004	1.08	1.18	1.06	1.17
0.006	1.60	1.75	1.57	1.68
0.008	2.13	2.34	2.09	2.12

[a] One ml of buffer is added to 10 ml gallic acid to maintain volume constant.

Gallic acid (10 ml) was kept in contact with 1.0 g enzyme-free and enzyme (tannase) entrapped polyacrylamide gel for 1 h on a gyratary shaker (120 rpm), and filtered. The absorbance of the filtrates was measured at 250 nm against 50 mM sodium acetate buffer (pH 4.7).

Kinetic parameters essential for a satisfactory enzymatic reaction (assay) have been determined. The time course of the reaction catalysed by the immobilized tannase was studied employing polyacrylamide gel, Duolite-S-762 and collagen membrane. The results graphically represented in figure 2 show that under the conditions used, the reaction rate is linear from 10 min to at least up to 40 min in all the cases. When the substrate concentration was increased, the rate of reaction increased proportionately at least up to 0.008% tannic acid in the case of collagen-bound enzyme and to 0.01% in the other two cases (figure 3). Higher concentrations of tannic acid are probably necessary to saturate the enzyme or for substrate to efficiently permeate the matrices, but lead to much higher blanks at either $A_{310\ \mathrm{nm}}$ or $A_{250\ \mathrm{nm}}$. The effect of immobilized tannase concentration on the reaction rate was studied by varying the concentration of matrix bound tannase and lead to a proportionate increase in the reaction rate with 0.008% tannic acid (figure 4). In most of these cases (figures 2, 3 and 4), the plots do not pass through the origin. This may be because there was a difference in $A_{250\ \mathrm{nm}}$ between experimental sample and the control at zero min. This was also noticed in the experiment on the effect of immobilized enzyme concentration on activity by Weetal and Detar (1974). However it does not vitiate the assay since the differences in A_{250} between 30 min and 10 min are used for calculation of activity.

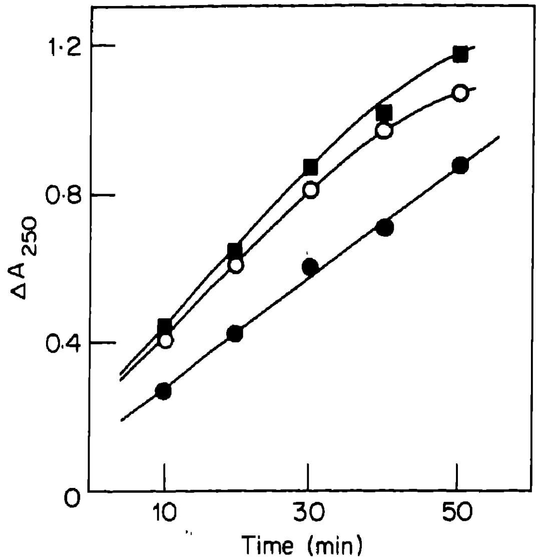

Figure 2. Time course of the reaction catalysed by immobilized tannase. Collagen-enzyme (10 mg, ■); Duolite-enzyme (200 mg, O); and polyacrylamide-enzyme (100 mg, ●). Ten ml of 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7) was added at 25°C. Blank contained adsorbant, without the enzyme.

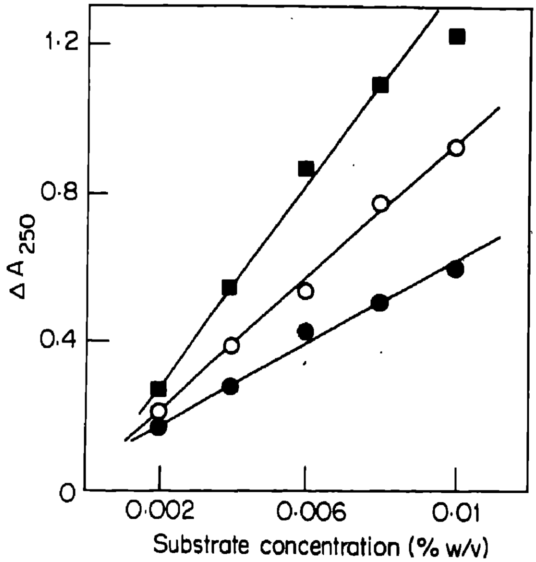

Figure 3. Effect of substrate concentration. Ten ml of substrate tannic acid in 50 mM sodium acetate buffer (pH 4.7) of various concentrations (0.002% to 0.01%) was added to tannase immobilized on collagen (50 mg, ■); Duolite (1 g, O) and polyacrylamide gel (1 g, ●). Incubation was for 20 min at 25°C.

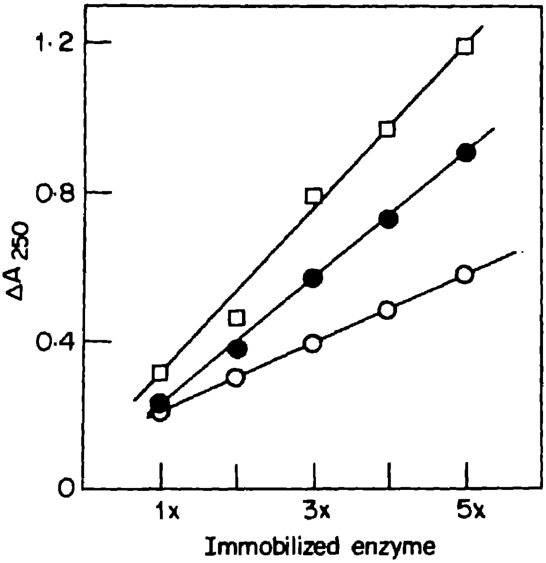

Figure 4. Effect of immobilized tannase at different concentrations. Collagen-enzyme (5 to 25 mg, □), Duolite-enzyme (20 to 100 mg, O) and polacrylamide gel enzyme (50 to 250 mg, ●). Ten ml of 0.008% tannic acid in 50 mM sodium acetate buffer (pH 4.7) was added. Incubation for 20 min at 25°C.

In the above cases, gallic acid was detectable as a product of hydrolysis by paper chromatography (figure 5) according to the procedure of Iibuchi *et al.* (1972) and the product had a UV absorption spectrum similar to that of gallic acid.

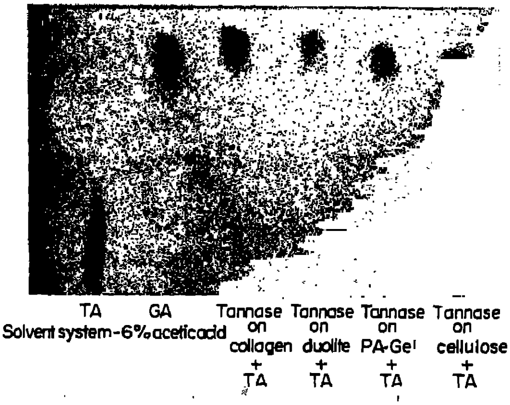

Figure 5. Paper chromatography of tannic acid and its hydrolyzed product by the action of immobilized tannase with standard gallic acid.

Ten ml of tannic acid (0.4%) in 50 mM sodium acetate buffer (pH 4.7) was added to tannase immobilized on collagen (200 mg), Duolite (2 g) and polyacrylamide gel (2 g.), incubated for 4 h at 25°C on a gyratary shaker (120 rpm), and filtered. The filtrates (20 µl) were spotted on Whatman (3 mm) chromatographic paper along with standard gallic (0.4%) and tannic acid (0.4%). Six % acetic acid was used as solvent system and 2% ferric chloride as the spraying agent.

Conclusions

On the basis of the above data, the following method of determination of immobilized tannase activity has been proposed. Enzyme bound matrix (50 mg collagen or 1 g Duolite or 1 g polyacrylámide gel; quantity can be reduced depending on the activity units retained in the matrix) and the same quantity of similarly treated enzyme free matrix are added separately to two 25 ml Erlenmeyer flasks containing 10 ml tannic acid (upto 0.01%) in 50 mM sodium acetate buffer (pH 4.7). The reaction mixtures are incubated for 20 min at 25° in a gyratary shaker (120 rpm). The filtrates obtained from the reaction mixture are read at 250 nm against the buffer. The ΔA_{250} is taken as a measure of immobilized tannase activity. A unit of activity has been defined previously. With chitin, 20-30% adsorption of product (gallic acid) was observed and hence this method is not likely to be satisfactory for the chitin-tannase complex. Tea tannins are also efficiently hydrolyzed by the action of tannase immobilized on polyacrylamide gel, Duolite-S-762 and collagen membrane. Gallic acid was identified as a product of hydrolysis by paper chromatography.

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Embryonic control of isocitrate lyase activity in cotyledons of germinating soybean

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Abstract. The activity of isocitrate lyase (EC 4.1.3.1) in the cotyledons of germinating soybean is controlled by the embryonic axis. Plant growth regulators like gibberellic acid, indole acetic acid and 2,4-dichlorophenoxy acetic acid are able to increase the enzyme activity in cotyledons of whole seedlings but not in dissected cotyledons. The control of induction of the enzyme activity during germination by the embryo could be mediated by the elaboration of kinetin.

Keywords. Isocitrate lyase; soybean; germination.

Introduction

The embryonic axis is known to control many events occurring in the cotyledons of germinating seeds (Penner and Ashton, 1967; Bilderback, 1974; Heimer *et al.*, 1976). However, the role of the embryo in controlling isocitrate lyase (EC 4.1.3.1) activity in cotyledons of germinating seeds is not clear. In castor bean endosperm, pea nut, *Cucumis sativa* and *Cucurbita maxima*, the embryo does not have a controlling function (Tanner and Beevers, 1965; Marcus and Feeley, 1964; Ford *et al.*, 1976), but in the case of squash melon and sunflower, cytokinins from the embryo control the appearance of the lyase activity in cotyledons (Penner and Ashton, 1967; Heimer *et al.*, 1976). A similar role has also been ascribed to gibberellins in wheat aleurone cells (Tavener and Laidman, 1975) and in pondrosa pine seeds (Bilderback, 1974). The present studies were conducted with a view to investigate the role of the embryo in the development of isocitrate lyase activity in germinating soybean seeds.

Materials and methods

Soybean (*Glycine max* L., variety Bragg) seeds were procured from the Department of Plant Breeding, Punjab Agricultural University, Ludhiana. Growth regulators such as gibberellic acid (GA_3), indole acetic acid (IAA), kinetin and other chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

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Abbreviations used: GA_3, Gibberellic acid; IAA, Indole acetic acid; 2,4-D, 2,4-Dichlorophenoxy acetic acid.

Germination

Before germination, the seeds were surface-sterilized with 0.1% mercuric chloride and washed with distilled water. The washed seeds were sown in petri plates (50 mm×20 mm) lined with filter paper saturated with distilled water at 25 ±1°C in a biological oxygen demand incubator. To study the effect of growth regulators and other chemicals distilled water was replaced with solutions of these compounds. Samples were collected at desired time, washed with distilled water and analysed.

Enzyme assay

The tissues were ground in a chilled mortar, in ice cold 0.05 M phosphate buffer, pH 7.6. The ratio of plant material to buffer was 1:5 (w/v). The slurry was filtered through 4 layers of muslin cloth and the filtrate was centrifuged at 15,000 *g* for 20 min. The clear supernatant was used as the enzyme preparation.

The enzyme was assayed according to the procedure of Gientika and Cherry (1968). The reaction mixture, in a total volume of 1.5 ml, contained—10 μM phosphate buffer, pH 7.6; 2.5 μM glutathione; 5 μM $MgSO_4$; 5 μM isocitrate (trisodium salt) and 0.1 ml of enzyme extract. The reaction was started by adding the substrate. After incubation for 15 min at 30°C, the reaction was terminated by adding 0.3 ml of 100% trichloroacetic acid. The denatured protein was removed by centrifugation. To 1 ml of the protein-free supernatant, 0.66 ml of 0.1% 2,4-dinitrophenyl hydrazine in 2 N HCl was added and the reaction mixture was kept at room temperature for 20 min. Then 3.35 ml of 2.5 N sodium hydroxide was added and the absorbance measured at 445 nm.

Protein estimation

Protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Results and discussion

The data on the effect of the embryo on the appearance of isocitrate lyase activity in cotyledons of germinating soybean are shown in table 1. The enzyme activity in dissected cotyledons was found to be only about 57% of that in cotyledons of whole seedlings germinating under similar conditions. Thus the data indicate that isocitrate lyase activity is partly under the control of the embryonic axis. These findings are contrary to the study of Marcus and Feeley (1964) in pea nut and Ford *et al.* (1976) in *Cucumis sativa* and *Cucurbita maxima* but are in agreement with the studies of Tavener and Laidman (1975), Jones (1972) and Bilderback (1974) in wheat, barley and ponderosa pine seeds respectively. The activity of isocitrate lyase was measured (table 1). The data in table 1 clearly indicate that the removal of the embryo during the first 3 days of germination markedly affects the subsequent appearance of the enzyme activity. But if the embryo was removed after 3 days of germination, it had no effect. It can therefore be concluded that the presence of the embryo is essential during the initial stages of germination and some factor(s) from embryo may be responsible for the observed increase in isocitrate lyase activity in the cotyledons.

Table 1. Effect of embryo on isocitrate lyase activity in cotyledons of germinating soybean.

Incubation		Specific activity
Intact seedlings (days)	Followed by excision of cotyledons and incubation (days)	μmol of glyoxylate released/mg protein/ 15 min
6 (intact cotyledons)	0	0.98
5	1	0.95
4	2	0.94
3	3	0.70
2	4	0.60
1	5	0.56
0 (dissected cotyledons)	6	0.56

The values are the average of 3 independent determinations.

Isocitrate lyase activity in the cotyledons of seeds germinated for 6 days in the presence of various concentrations of GA_3, IAA, 2,4-D and kinetin is recorded in table 2. All the four growth regulators tested increased the lyase activity in cotyledons. The optimal concentration of GA_3 was 10 μM (which increased the activity by 41%) while that of kinetin was 100 μM at which a 37% increase in activity was observed. IAA and 2,4-D both had an optimal concentration of 1 μM. Similar effects of plant growth regulators have also been observed in hazel (Pinfield, 1968; Potempta and Galsky, 1973), *Pinus pinea* (Martinez *et al.*, 1975) and ponderosa pine (Bilderback, 1974) seedlings.

Table 2. Effect of growth regulators on isocitrate lyase activity in cotyledons of germinating soybean.

Concentration (μM)	Specific activity [a]			
	GA_3	IAA	Kinetin	2,4-D
Control	1.0	1.0	1.0	1.1
0.1	1.0	1.2	1.1	1.4
1.0	1.2	1.4	1.1	1.5
10	1.5	1.4	1.2	1.3
100	1.2	1.0	1.4	1.2
1000	1.1	1.0	—	—

[a] μmol of glyoxylate released/mg protein/15 min.

The values are the average of 3 independent observations.

Data on the effect of the interaction of growth regulators on the induction of isocitrate lyase activity are shown in table 3.

Table 3. Effect of interaction of growth regulators on isocitrate lyase activity in cotyledons of germinating soybean.

Growth regulators (μM)	Specific activity (μmol of glyoxylate released/ mg protein/15 min)
Control	1.0
GA_3 (10)	1.4
IAA (1)	1.4
Kinetin (100)	1.4
Kinetin (100) + GA_3 (10)	1.6
Kinetin (100) + IAA (1)	1.4
GA_3 (10) + IAA (1)	1.5

The values are the average of 4 independent determinations.

In an experiment to ascertain the growth regulatory nature of the embryonic factor(s), excised cotyledons were incubated for 6 days in solutions of GA_3, IAA, kinetin and 2,4-D and the isocitrate lyase activity was measured (table 4). Kinetin increased the lyase activity to a considerable extent but could not completely replace the embryo in this respect. So it appears likely that kinetin may be one of

Table 4. Effect of growth regulators on isocitrate lyase activity in dissected soybean cotyledons.

Growth regulator	Concentration (μM)	Specific activity (μmol of glyoxylate released/ mg protein/15 min)
Control (intact cotyledons)		1.0
Control (excised cotyledons)		0.6
GA_3	10	0.6
IAA	1	0.6
Kinetin	250	0.9
2,4-D	1	0.5

The values are the average of 3 independent determinations.

the factors produced by the embryo. GA_3 could increase the enzyme activity only to a small extent while IAA and 2,4-D failed to do so. This suggests strongly that GA_3, IAA and 2,4-D increase the isocitrate lyase activity in cotyledons of germinating seeds (table 2) in an indirect manner and by themselves are without any effect. In squash cotyledons, benzyl adenine could partially replace the embryonic factors (Penner and Ashton, 1967) while in wheat aleurone cells, GA_3 was effective in increasing isocitrate lyase activity (Tavener and Laidman, 1975).

Acknowledgements

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Cardiotoxin of the Indian cobra *(Naja naja)* is a pyrophosphatase

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Abstract. An inorganic pyrophosphatase has been purified to apparent homogeniety from Indian cobra (*Naja naja*) venom, with a ten-fold increase in specific activity. The enzyme activity is intrinsic to a protein fraction in the venom which is normally termed cardiotoxin, cobramine, cytotoxin and so on. The enzyme shows a low K_m (70 μM) and high heat stability. The enzyme was active against sodium pyrophosphate; it also hydrolyses a few mononucletides and sugar phosphates at much lower rates. The physiological significance of inorganic pyrophosphatase in venom is discussed.

Keywords. Cobra venom; *Naja naja*; cardiotoxin; pyrophosphatase.

Introduction

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, E.C.3.6.1.1) is not a hitherto acknowledged constituent of Indian cobra (*Naja naja*) venom, which is known to contain many other enzymes (Jimenez-Porras, 1970). Amongst the known enzymes, are two which have the ability to hydrolyze the pyrophosphate linkage in ATP, NAD^+ and coenzyme A (Kaye, 1960; Jimenez-Porras, 1970). We had earlier noted the presence of inorganic pyrophosphatase activity in both crude venom and in several basic protein fractions obtained from the venom (Achyuthan and Ramachandran, 1980). In this paper, we report the pyrophosphatase nature of one of those proteins (Fraction X), which is none other than cardiotoxin, a well-known non-neurotoxic protein of cobra venom.

Materials and methods

Crude venom of Indian cobra (*Naja naja*) (Batch No. 195-210) was obtained from Haffkine Institute, Bombay. Cobramine B (twelve year-old sample) was a gift from Dr. J. Wolff of the National Institute of Health, Bethesda, Maryland, USA. All other chemicals used were of analytical grade. All solutions were made in glass-distilled water.

Isolation and purity of Fraction X

Cobra venom was fractionated on columns of CM-Sephadex C-25 as described previously (Achyuthan *et al.*, 1980). Fraction X, the protein under study, is highly basic and eluted as the penultimate peak under the chromatographic conditions used. It had been found to be homogeneous on rechromatography and disc gel electrophoresis under conditions earlier described (Achyuthan *et al.*, 1980).

Enzyme assay

Inorganic pyrophosphatase activity was assayed according to Heppel (1955), using sodium pyrophosphate as substrate. The reaction mixture contained 0.5 mg enzyme protein, 1 μmol of pyrophosphate and 1.1 μmol of $MgCl_2$ in a final volume of 1.0 ml of 0.05 M Tris-HCl buffer, pH 7.2. At the end of 15 min of incubation (at 37° ±1°C), the inorganic phosphate liberated was estimated by the method of Fiske and Subbarow (1923). In all assays, appropriate corrections for controls were made. The specific activity of pyrophosphatase is defined as micromol of phosphate liberated per minute per mg of protein.

As traces of 5′-nucleotidase activity had been earlier found (Achyuthan and Ramachandran, 1980) in Fraction X, assays for nucleotidase activity at optimal substrate concentration (ten-fold higher, relative to pyrophosphate) were done. Phosphatase assays also were done at a ten-fold higher substrate concentration.

K_m *and* V_{max}

The Michaelis constants were calculated according to the direct linear plot of Eisenthal and Cornish-Bowden (1974).

Results

Purification and properties

Fraction X with the pyrophosphatase activity is obtained in an yield of 15.4% of the weight of crude venom. The specific activity (V_{max} under standard conditions) corresponded to 0.12 I.U. indicating that a ten-fold purification had been achieved by fractionation of the crude venom. The enzyme activity in solution is stable for upto one month if kept in the frozen state. A twelve year-old Cobramine B sample (Larsen and Wolff, 1967) stored in the lyophilized state still displayed 50% of the activity shown by Fraction X. The enzyme has a broad pH optimum (pH 6.5-7.5). About 50% of this activity was evident even at the lower pH of 5.5 and the higher pH of 9.5. The reaction was zero order upto 50% hydrolysis of substrate.

Substrate and other effects

Above a final concentration of 6 mM sodium pyrophosphate the activity decreases and is about 40% at a pyrophosphate concentration of 16 mM (figure 1). Otherwise, the activity is constant over the range of substrate concentration, 0.2-6 mM.

The effect of metal ions, tris, chelators, thiols and some anions is shown in table 1. High concentrations of many metal ions and anions inhibit the enzyme activity to varying extents. The activity is stable over a broad range of buffer (tris) concentrations, full activity being retained even upto 0.8 M tris. Chelating agents, thiols and reagents for thiol groups have no effect on enzymatic activity (table 1). The protein contains no thiol groups as can be ascertained by reaction with *p*-hydroxymercuribenzoate by the method of Boyer (1954).

The enzyme is optimally active between 40°C and 60°C, below and above which the activity declines. The enzyme activity is remarkably stable, withstanding

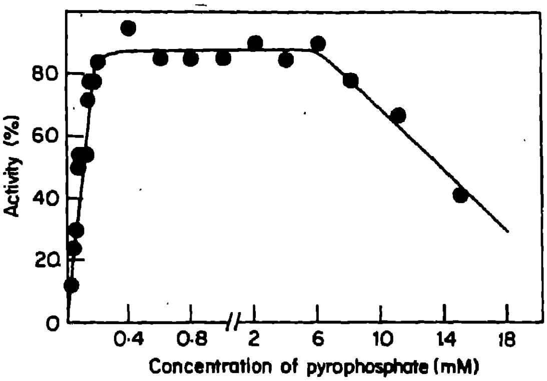

Figure 1. Effect of substrate concentration on enzyme activity. The enzyme (0.5 mg) was incubated with varying concentrations of sodium pyrophosphatase (upto 15 µmol/ml). Other conditions of incubation are described in the text. At the end of 15 min the inorganic phosphate liberated was estimated.

Table 1. Effect of metal ions, anions, tris, chelators, thiols and thiol reagents on *Naja naja* pyrophosphatase

Ingredient	Concentration (mM)	Inhibition* (%)
Metal ions:		
Cu^{2+} and Ag^{+}	0.5	65-73
	10	70-88
Ca^{2+}	0.5	68
	10	83
Zn^{2+}, Cd^{2+} and Hg^{2+}	0.5	70-75
	10	77-90
Mn^{2+}	0.5	57
	10	69
Ni^{2+} and Co^{2+}	0.5	24-73
	10	57-77
Anions:		
WoO_4^{2-}	10	17
MoO_4^{2-}	10	24
Oxalate	10	10
F^-	10	82
CN^-	10	50

Thiols (cysteine, reduced glutathione, and dithiothreitol) at 10 mM have no effect on activity at pH 7.0. N-Ethylmaleimide and iodoacetate (0.01 to 10 mM) have no effect.

Tris-HCl (pH 7.5), upto 0.8 M and the metal chelators, 8-hydroxyquinoline, and α, α'-bipyridyl and ethylene-diaminetetraacetate, at concentrations upto 10 mM have no effect on enzyme activity.

* Values for Zn^{2+}, Cd^{2+}, Cu^{2+} and Co^{2+} and for WoO_4^{2-} and MoO_4^{2-} are corrected for protein precipitation. Mg^{2+} (1-10 mM) has no effect on activity.

heating at 60°C upto 30 min without any loss in activity (figure 2). It also retains 80% of its activity after heating for 10 min at 90°C, after which time however, the enzyme is rapidly inactivated (figure 2). The Q_{10} values obtained are 1.8 (20°C-30°C) and 1.1 (30°C-40°C). The energy of activation calculated from the Arrhenius plot is 10.12 Kcal/mol.

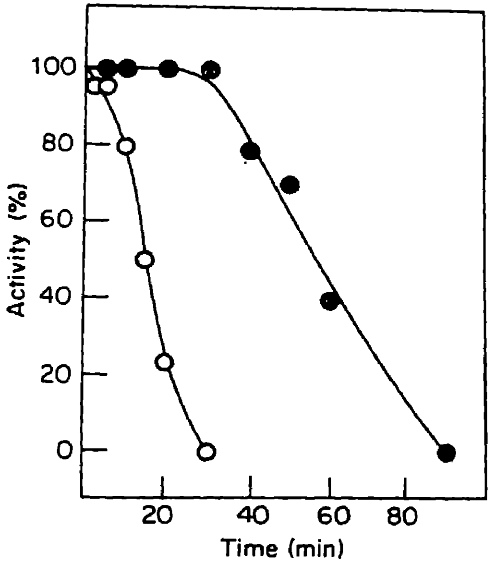

Figure 2. Effect of heat treatment at 60°C (●) and 90°C (O) on enzyme activity. The enzyme solution was heated in a water bath at the desired temperature (±1°C) for different durations. The solutions were taken out, cooled rapidly and assayed for pyrophosphatase activity as described in the text.

Substrate specificity

Besides pyrophosphate, the enzyme also hydrolyzes a few nucleotides and sugar phosphates, though at markedly reduced rates (table 2). The K_m value for hydrolysis of UMP (uridylic acid) was 100-fold higher than that for pyrophosphate and the corresponding V_{max} was only a third.

Discussion

No studies of inorganic pyrophosphatases in cobra venoms exist. Johnson *et al.* (1953) had observed the existence of very weak inorganic pyrophosphatase activity in cobra venom. They had observed that one mg of venom will hydrolyze 1.5 μmol of pyrophosphate in *six days*. The prolonged incubation period would make one wonder whether the observed activity was intrinsic to venom. The present study demonstrates beyond doubt the existence of inorganic pyrophosphatase in cobra venom. The enzyme activity is associated with a basic protein which is also a toxin.

The pyrophosphatase described here is "neutral" and displays a broad pH optimum of 6.5-7.5. Inorganic pyrophosphatases with acidic, neutral and alkaline

Table 2. Organic phosphates and pyrophosphate as substrates for *Naja naja* pyrophosphatase

Compound	Effectiveness*
Pyrophosphate	100
Cytidylic acid (CMP −3′)	25
Adenylic acid (AMP −5′)	25
Uridylic acid (UMP −5′)	22
Guanylic acid (GMP −5′)	25
Glucose−1−phosphate	13
Glucose−6−phosphate	13
Others**	0

* The effectiveness of the enzyme on sodium pyrophosphate under standard assay conditions is taken as 100. Under standard assay conditions, other substrates are scarcely attacked. The values entered in the table are obtained at ten-fold higher substrate concentration (see text).

** Several other organic phosphates (viz. thymidylic acid (TMP −5′), cyclic−AMP, adenylic acid (ATP −5′), cytidylic acid (CTP −5′), inosinic acid (ITP −5′), uridylic acid (UTP −5′ and UDP −5′), cofactors (FMN, FAD, NADP, TPP), glycerophosphates (α− and β−), fructose −1, 6 −diphosphate and p-nitrophenyl phosphate) which were tested were not hydrolyzed.

pH optima are known (Butler, 1971; Josse and Wong, 1971). The Michaelis constant (70 μM) is quite low and indicates high affinity of the anionic substrate for the cationic enzyme protein. Fraction X (Cardiotoxin II) is a highly basic protein. It is possible that basic amino acid residues in the protein are involved in substrate binding. A similar high affinity for substrate has been observed for several other pyrophosphatases (Bailey and Webb, 1944; Naganna *et al.*, 1955). Unlike pyrophosphatases of yeast, brain and rat liver which are inactive at pyrophosphate concentrations of 0.5-3 mM, the venom enzyme remains unaffected at concentrations upto 6 mM.

Most pyrophosphatases have an absolute requirement for divalent metal ions, usually Mg^{2+}, for activity (Butler, 1971; Josse and Wong, 1971). Cobra venom pyrophosphatase (Fraction X) is neither activated by added Mg^{2+} nor inhibited by ethylenediaminetetraacetate (EDTA) (table 1). Varying the ratio of the concentrations of Mg^{2+} to pyrophosphate widely in an effort to enhance enzyme activity turned out to be of no consequence. High concentrations (1-10 mM) of two other chelating agents (table 1) have no inhibitory effect. Fluoride (10 mM) causes 82% inhibition of the enzyme. Instances are known where inhibition by fluoride is related to removal of essential cations such as Ca^{2+} and Mg^{2+}, or to formation by F^- of complexes with substrates or products which then inhibit the enzyme.

The inhibition by other metal ions and anions (table 1) follows set patterns reported by earlier workers (Naganna *et al.*, 1955; Butler, 1971; Josse and Wong,

1971) and in part may be accounted for by protein precipitation, complexing with pyrophosphate and other ill-defined interactions. Inhibition by cyanide amounts to 50%, and this may have to do with the stability of four disulfide bonds which are known to be present in the protein of Fraction X (Srinivasa, Achyuthan and Ramachandran, unpublished data) which lacks thiol groups. Thiols and reagents for thiol groups have generally no effect on enzyme activity (table 1). Thus, cobra venom pyrophosphatase is not an −SH enzyme, unlike some known pyrophosphatases (Butler, 1971).

Cobra venom pyrophosphatase (Fraction X) is remarkably stable to heat (figure 2). Cardiotoxin II (Cobramine B) activity also is known not to suffer attrition in toxicity on heating for moderate durations (Larsen and Wolff, 1967). In so far as heat stability is concerned, Fraction X shares this property with the pyrophosphatases of *E. coli* studied by Josse & Wong (1971) and of *Proteus vulgaris* studied by Swartz *et al.* (1958).

Pyrophosphatases studied hitherto show varying degrees of substrate specificity (Butler, 1971; Josse and Wong, 1971). The venom enzyme also attacks at slow rates some mononucleotides and two sugar phosphates (table 2).

What is intriguing is that cobra venom pyrophosphatase (Fraction X) is also a toxin in its own right. (Achyuthan *et al.*, 1980; Achyuthan and Ramachandran, 1980). It closely resemble the group of basic polypeptides severally named cardiotoxin, cytotoxin and cobramines (Jimenez-Porras, 1970). All these are related (similar, if not identical) polypeptides having low toxicity and strong cardiotoxic activity (Jimenez-Porras, 1970). Evidence based on a variety of chemical, physical, biological and enzymatic properties of Fraction X and Combramine B points to their close identity (Achyuthan *et al.*, 1980). The amino acid composition of Fraction X is also similar to that reported for the cardiotoxin group of polypeptides (Srinivasa, Achyuthan and Ramachandran, unpublished data). The pronounced inotropic effect of Fraction X on isolated ventricle muscle is similar (unpublished data) to that of cardiotoxins. Disc electrophoresis of Fraction X, Cobramine B and a mixture of the two proteins show identical mobilities at pH 4.2 and 6.0 (figure 3), and at pH 7.5 and 8.0 (not shown). Both Fraction X and a twelve year-old sample Cobramine B, isolated by Larsen and Wolff (1967) by a different procedure, and tested by us, display pyrophosphatase activity, with the latter retaining half as much specific activity (0.06 I.U./mg) as the former. The name cardiotoxin seems appropriate for protein fractions IX and X, in view of the well-known cardiovascular effects produced by such basic polypeptides of venom, and for future reference we would like to identify the later eluting protein fraction, X, as Cardiotoxin II.

Whether the pyrophosphatase activity is causally related to the toxicity of cardiotoxin is hard to say that the moment. Also, the physiological significance if any, of inorganic pyrophosphatase *per se* in venom is not quite clear. Fractions IX and X, which are active in this manner, account for nearly one third the weight of crude venom. Venom pyrophosphatase can indeed contribute to near complete hydrolysis of certain important cellular constituents like ATP and ADP by the respective hydrolytic enzymes present in venom, by further hydrolysis of pyrophosphate released as product by such enzymes. This would naturally deplete the of vital compounds.

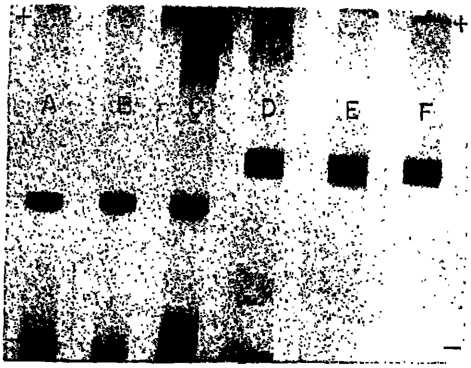

Figure 3. Disc electrophoresis of Fraction X, Cobramine B and a mixture of the two prote at pH 4.2 (A, B and C) and at pH 6.0 (4, 5 and 6). Electrophoresis was carried out on 7.5% g with tris-glycine (pH 8.5) as the matrix buffer. Current: 4 mA/tube.

Electrolyte: 0.2 M acetate buffer (pH 4.2) or 0.2 M citrate phosphate buffer (pH 6.0). Ti of run: 60 min (for 1, 2 and 3 gels) and 90 min (for gels 4, 5 and 6). The gels were stained fc min in 1% Amido Black 10B in 7% acetic acid and destained with 7% acetic acid.

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Transport of retinol in the duck plasma

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Abstract. Retinol-binding protein and prealbumin were isolated from duck plasma by chromatography on DEAE-cellulose and DEAE-Sephadex A-50, gel filtration on Sephadex G-100 and preparative polyacrylamide gel electrophoresis. The molecular weights of the retinol-binding protein-prealbumin complex, prealbumin and retinol-binding protein were found to be 75,000, 55,0000 and 20,000, respectively. On sodium dodecyl sulphate polyacrylamide gel electrophoresis, prealbumin dissociated into identical subunits exhibiting a molecular weight of 13,500. Retinol-binding protein exhibited microheterogeneity on electrophoresis, whereas prealbumin moved as a single band unlike the multiple bands observed in chicken and rat. The ultraviolet and fluorescence spectra of the two proteins were similar to those isolated from other species. No carbohydrate moiety was detected in either retinol-binding protein or prealbumin. Duck retinol-binding protein and prealbumin showed cross-reactivity with their counterparts in chicken but differed immunologically from those of goat and man. Retinol-binding protein and prealbumin could be dissociated at low ionic strength, in 2M urea, by CM-sephadex chromatography or on preparative electrophoresis. Although the transport of retinol in duck plasma is mediated by carrier proteins as in other species, it is distinguished by the absence of microheterogeneity in prealbumin and of an apo-retinol-binding protein form that could be transported in the plasma.

Keywords. Retinol; prealbumin; plasma transport; duck.

Introduction

It is well established that retinol is transported in human plasma bound to a specific retinol-binding protein (Kanai *et al.*, 1968). The retinol-binding protein forms a tight complex with plasma prealbumin and normally circulates as an equimolar complex with it (Peterson and Berggard, 1971; Raz and Goodman, 1969; Peterson, 1971). A similar retinol-transport system has been reported in several other species (Muto *et al.*, 1973; Peterson *et al.*, 1974; Abe *et al.*, 1975; Bhat *et al.*, 1977) and evidence has been presented for the identity of the plasma and egg-yolk retinol-binding protein-prealbumin complexes in the case of chicken (Heller, 1976). In pursuance of earlier studies in our laboratory on the mode of transport of retinol for embryonic development in duck (Sreekrishna and Cama, 1978), the plasma transport system in duck was characterized by the isolation of retinol-binding protein and prealbumin.

Materials and methods

Materials

Blood was collected after decapitation of the ducks and was allowed to clot. The clear supernatant serum was collected and processed immediately. DEAE-cellulose (0.9 milliequivalents/g), Sephadex G-100, bovine serum albumin, ovalbumin, cytochrome C, α-chymotrypsinogen, hexokinase, acrylamide, N,N,N′,N′-tetramethylene diamine, ammonium persulphate, bromophenol blue, Coomassie Brilliant Blue, Tris (hydroxymethylaminomethane) and glycine were all purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Agar and Freund's complete adjuvant were obtained from Difco Lab., Detroit, Michigan, USA. All other chemicals were of reagent grade.

Methods

Protein-bound retinol was monitored by measuring the relative fluorescence intensity (*F*) at 470 nm upon excitation at 330 nm in a Perkin Elmer spectrofluorometer (Model 203). Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard. Analytical polyacrylamide gel (7.5% w/v) electrophoresis was performed by the method of Davis (1964). Glycoprotein staining was performed according to the method of Segrest and Jackson (1972). Preparative electrophoresis was carried out using cylindrical gels (1.2×9 cm) with 0.5-1.0 ml of spacer gel. After electrophoresis, the gel was examined under an ultraviolet lamp for fluorescence of protein-bound retinol. The fluorescent zone was sliced and retinol-binding protein eluted from the gel slice by electro-elution or by homogenization and extraction with 20mM Tris-HCl buffer, pH 7.6 containing 0.2M NaCl. The gel particles were removed either by centrifugation at 12,000 *g* for 10 min or by filtration through a Whatman No. 1 filter paper. For prealbumin, the region of the gel between the fluorescent zone and the marker dye was cut into thin slices and the protein eluted from each slice as described above. The single protein peak obtained with a mobility intermediate between the marker dye and albumin was designated as prealbumin. Occassionally, the prealbumin band was identified by comparison with a duplicate gel stained with Coomassie Brilliant Blue G.

Determination of molecular weight. Molecular weights were determined by gel filtration using a Sephadex G-100 column calibrated with cytochrome C, α-chymotrypsinogen, ovalbumin, bovine serum albumin and hexokinase according to the method of Whitaker (1963). Sodium dodecyl sulphate Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) using bovine serum albumin, ovalbumin and cytochrome C as standards.

Preparation of antibodies. Rabbits (Institute strain) were immunized by intradermal injection, once a week, with 100 μg of retinol-binding protein or prealbumin in phosphate buffered saline emulsified in Freund's complete adjuvant. After three immunizations, a booster injection of 300 μg of protein in phosphate buffered saline was given and after six days the animals were bled through the marginal ear vein, the serum prepared and stored frozen. Immuno-double diffusion was carried out according to the method of Ouchterlony (1967).

Binding experiments. The binding of thyroxine and retinol-binding protein to prealbumin was monitored by the quenching of the protein fluorescence of prealbumin. The control employed in studying thyroxine binding was N-acetyl-L-tryptophanamide having the same absorbance as that of prealbumin. When the binding of retinol-binding protein was being monitored, the increase in the fluorescence upon addition of retinol-binding protein to buffer alone was deducted from the experiment.

The dissociation-association experiments were carried out by gel filtration on a Sephadex G-100 column (0.9×38 cm).

Preparation of apo-retinol-binding protein. Apo-retinol-binding protein was prepared by shaking retinol-binding protein solution with an equal volume of diethyl ether at a rate of 75 ± 5 strokes per min in the dark at 25 ±2°C for 6 h, under nitrogen in a stoppered test tube.

Results

Purification

Duck serum was applied onto a DEAE-cellulose column and the bound protein were eluted by a linear gradient of sodium chloride concentration from 0.1 to 0.35M. Protein-bound retinol eluted on the descending side of the main protein peak (figure 1). The fluorescent fractions were pooled, dialyzed against 0.1M

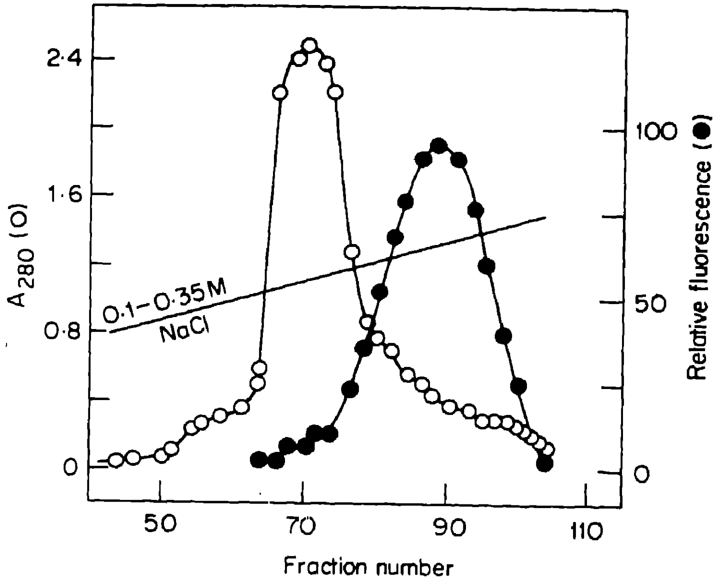

Figure 1. DEAE-cellulose chromatography of duck serum. Serum (100ml) dialyzed against 20 mM Tris-HCl buffer pH 7.6, containing 0.1 M NaCl was loaded onto a column (2×50 cm) of DEAE-cellulose equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of sodium chloride from 0.1 to 0.35 M. Fractions of 10 ml were collected at a flow rate of 30 ml/h. Fluorescent fractions were pooled.

sodium acetate buffer, pH 5.4, and subjected to a second ion exchange chromatography on DEAE-Sephadex. Elution was carried out by a linear gradient of 0.1 to 0.5M sodium acetate. Sephadex G-100 gel filtration of the fluorescent fraction from the above step resulted in the elution of two protein peaks (figure 2). The fluorescence was associated with the second peak. These fractions were pooled, concentrated and subjected to preparative polacrylamide gel electrophoresis. Localization of proteins and elution was carried out as described under Methods. Retinol-binding protein, after this step, was occasionally found to be contaminated with traces of albumin and hence was further purified by gel filtration on Sephadex G-100. The prealbumin obtained was pure and did not require further purification. Retinol-binding protein and prealbumin could be reassociated to form a complex (see below).

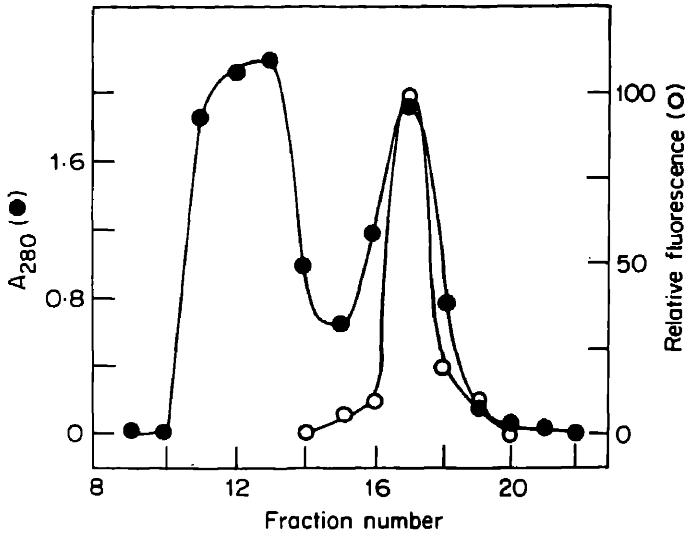

Figure 2. Sephadex G-100 gel filtration. The fluorescent fraction from the ion exchange chromatography was concentrated, dialyzed against 20 mM Tris-HCl buffer pH 7.6, containing 0.2M NaCl and applied on a column (2×44 cm) of Sephadex G-100 equilibrated with the same buffer. Fractions of 3 ml were collected at a flow rate of 12 ml/h.

Electrophoretic pattern

On analytical polacrylamide gel electrophoresis, retinol-binding protein exhibited two closely spaced bands exhibiting greenish blue fluorescence characteristic of retinol. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated that the two species had the same molecular weight (M_r 20,000). In analogy with the existing nomenclature, the fast and slow moving bands were designated as holo$_f$-retinol-binding protein and holo$_s$-retinol-binding protein, respectively. Evidently, the amount of the fast moving spcies is much higher. Prealbumin exhibited a single band, with a much faster mobility, trailing just behind the marker dye (figure 3).

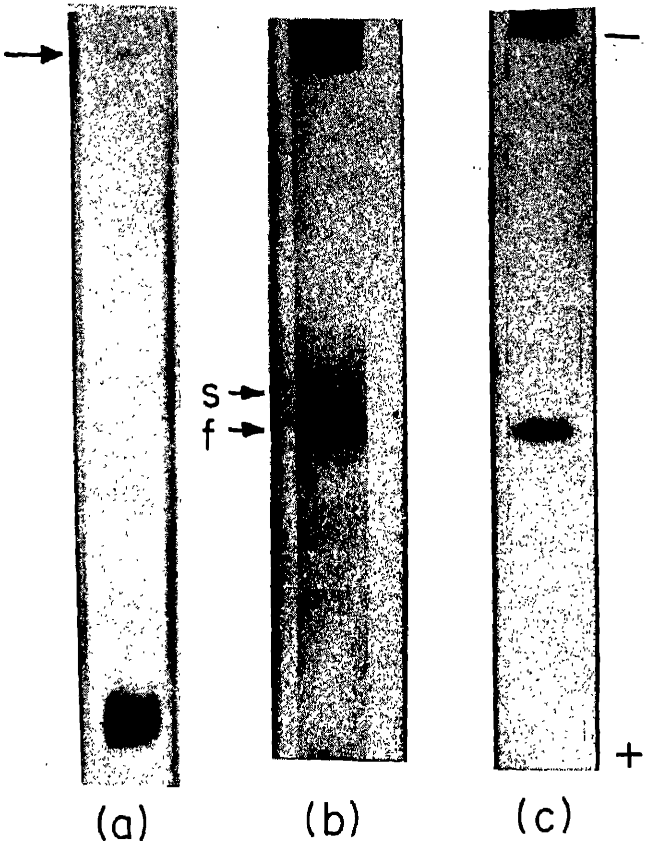

Figure 3. Electrophoretic pattern on 7.5% polyacrylamide gels in Tris-glycine buffer at pH 8.3 (a) Prealbumin (b) Retinol-binding protein. s and f indicate holo$_s$- and holo$_f$-retinol-binding proteins, respectively. (c) Holo$_f$-retinol-binding protein separated by preparative polyacrylamide gel electrophoresis.

Molecular weights

The molecular weights of complexed retinol-binding protein, prealbumin and retinol-binding protein, as estimated by gel filtration, were 75,000, 55,000 and 20,000, respectively. On sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, retinol-binding protein exhibited a molecular weight of 20,000 while prealbumin dissociated into subunits of molecular weight, 13,500.

Spectroscopic properties

Retinol-binding protein exhibits two absorption maxima in the ultraviolet regi one at 280 nm and the other at 330 nm. The retinol-binding protein-prealbun complex shows only a slight absorbance at 330 nm. The ratio of the absorbance 280 nm to 330 nm was found to be 1.2 in the case of retinol-binding protein and in the case of the retinol-binding protein-prealbumin complex. The fluorescen spectra of free as well as complexed retinol-binding protein exhibited t excitation maxima, one at 280 nm and the other at 330 nm and two emissi maxima, one at 330 nm and the other at 470 nm. Holo$_S$- and holo$_P$-retinol-bindi proteins were both fluorescent and their fluorescence spectra were similar ar overlapping.

Glycoprotein staining was found to be negative for retinol-binding protein well as prealbumin.

The addition of retinol-binding protein or L-thyroxine to prealbumin quenche the protein fluorescence indicating that both L-thyroxine and retinol-bindin protein could bind prealbumin (figure 4). Retinol-binding protein from whic retinol was extracted by diethylether was not able to bind prealbumin.

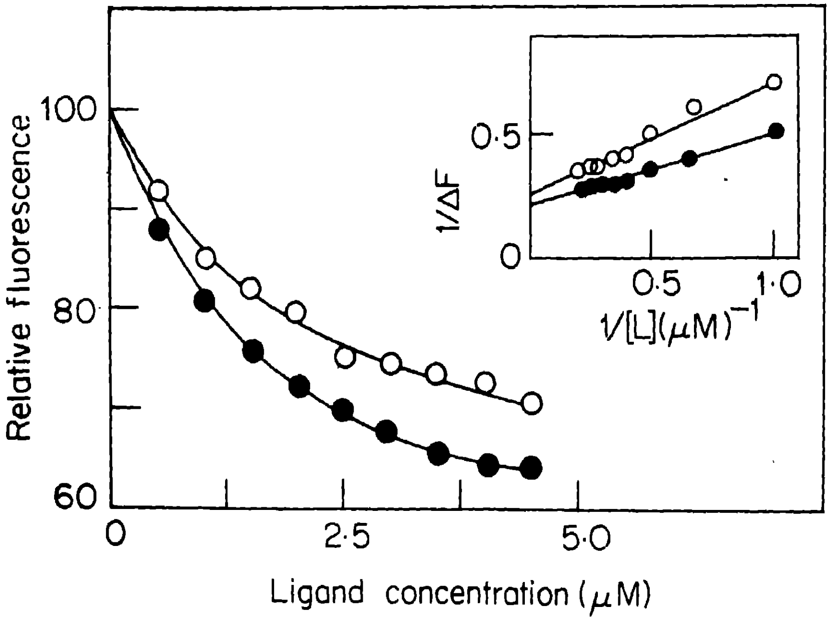

Figure 4. Quenching of the fluorescence of prealbumin by thyroxine and retinol-binding protein. Excitation and emission were set at 280 nm and 340 nm, respectively. To 1 μM solution of prealbumin in 20mM Tris-HCl buffer pH 7.6 containing 0.2M NaCl, aliquots of retinol-binding protein in the same buffer or L-thyroxine in 10mM NaOH were added. During titration with retinol-binding protein (O), the increase in fluorescence of the buffer alone on the addition of retinol-binding protein was deducted from the experiment. In the case of the titration with thyroxine (●), N-acetyl-L-tryptophanamide solution having the same absorbance as that of prealbumin was employed as blank. ΔF: Change in fluorescence. The ΔF_{max} obtained was 45 for thyroxine and 30 for retinol-binding protein.

Dissociation and association

The effects of various factors on the stability of the retinol-binding protein-prealbumin complex were tested. When buffers of various ionic strengths were tested, it was found that a low ionic strength buffer i.e., 2mM Tris-HCl, pH 7.6, could completely dissociate the complex. The complex was found to be less susceptible to changes in pH in the range of 8 to 3. A negligible amount of retinol-binding protein was found to be dissociated at pH 3.0. Another effective method of dissociation was chromatography on CM-Sephadex at pH 5.4, when the prealbumin eluted earlier than retinol-binding protein (figure 5). The presence of 2M urea in the buffer could also bring about dissociation.

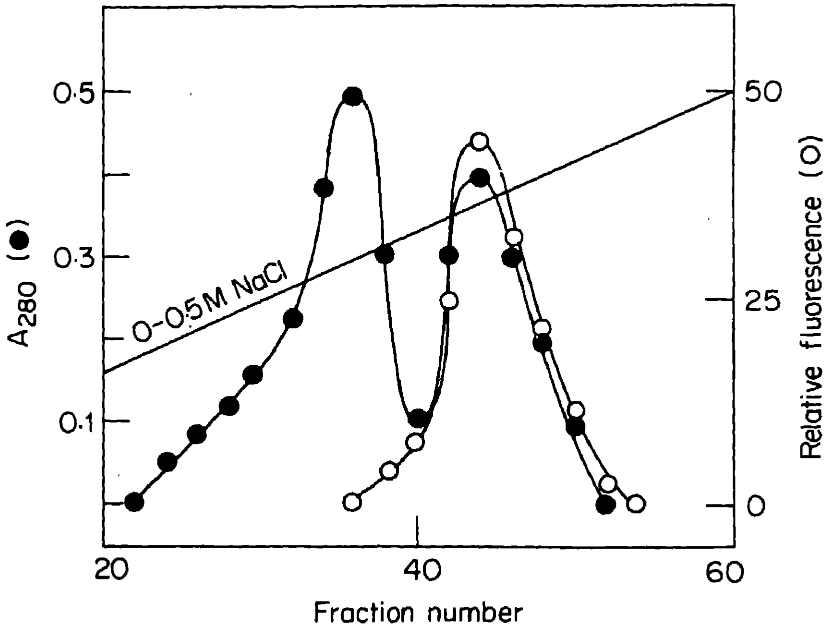

Figure 5. Dissociation of retinol-binding protein-prealbumin complex by CM-Sephadex chromatography. The complexed retinol-binding protein was dialyzed against 50 mM sodium acetate buffer, pH 5.4 and applied onto a column (1.4×49 cm) of CM-Sephadex equilibrated with the same buffer. Elution was carried out with a linear gradient of NaCl from 0 to 0.5M. Fractions of 5 ml were collected at a flow rate of 30 ml/h. Peak I was found to be prealbumin and peak II, retinol-binding protein by polyacrylamide gel electrophoresis.

The dissociation brought about by any of the above methods (including electrophoresis) was completely reversible. When retinol-binding protein and prealbumin were mixed and applied on a Sephadex G-100 column, the elution profile showed a shift of retinol fluorescence to an elution volume corresponding to a molecular weight of 75,000. No association was observed when prealbumin and apo-retinol-binding protein were applied on the column.

Immunological cross-reactivity

Ouchterlony immuno-double diffusion studies showed that duck retinol-binding protein and prealbumin exhibited strong cross-reactivity with their counterparts in chicken but did not show any cross reactivity with those of heterologous species like man and goat.

Discussion

In aggreement with the results obtained from other animal species, the transport of retinol in duck plasma is mediated by a retinol-binding protein-prealbumin complex. The physico-chemical properties of duck retinol-binding protein and prealbumin compare with those from other sources to some extent.

Duck retinol-binding protein showed microheterogeneity upon electrophoresis exhibiting a slow and a fast moving species. Abe *et al.* (1975) found chicken retinol-binding protein to be devoid of microheterogeneity while others (Kopelman *et al.*, 1976) reported to the contrary. We found, in the case of duck, two closely spaced bands of holo-retinol-binding protein but not of the apo-retinol-binding protein.

The A_{280}: A_{330} ratio of the duck retinol-binding protein is lower than that obtained in goat (Sreekrishna and Cama, 1979) and canine (Muto *et al.*, 1973) sources and agree well with the values reported in chicken (Heller, 1976).

An important observation in this study was that duck prealbumin did not exhibit microheterogeneity while that from other sources like chicken (Bhat *et al.*, 1977) exhibited multiple bands on electrophoresis. Another major difference between the duck apo-retinol-binding protein and that isolated from other species (Rask *et al.*, 1971) was its inability to bind prealbumin.

These results, while highlighting the universality of the carrier-mediated retinol-transport mechanism, emphasize that major differences exist in the physico-chemical and immunological properties of these proteins from several species.

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Allosteric serine hydroxymethyltransferase from monkey liver: Correlation of conformational changes caused by denaturants with the alterations in catalytic activity

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Abstract. The far-ultraviolet region circular dichroic spectrumof serine hydroxymethyltransferase from monkey liver showed that the protein is in an α-helical conformation. The near ultraviolet circular dichoric spectrum revealed two negative bands originating from the tertiary conformational environment of the aromatic amino acid residues. Addition of urea or guanidinium chloride perturbed the characteristic fluorescence and far ultraviolet circular dichroic spectrum of the enzyme. The decrease in $(\theta)_{222}$ and enzyme activity followed identical patterns with increasing concentrations of urea, whereas with guanidinium chloride, the loss of enzyme activity preceded the loss of secondary structure. 2-Chloroethanol, trifluoroethanol and sodium dodecyl sulphate enhanced the mean residue ellipticity values. In addition, sodium dodecyl sulphate also caused a perturbation of the fluorescence emission spectrum of the enzyme. Extremes of pH decreased the $-(\theta)_{222}$ value. Plots of $-(\theta)_{222}$ and enzyme activity as a function of pH showed maximal values at pH 7.4-7.5. These results suggested the prevalence of "conformational flexibility" in the structure of serine hydroxymethyltransferase.

Keywords. Serine hydroxymethyltransferase; conformational changes; denaturants; fluorescence; circular dichroism; structure-activity relationship.

Introduction

Earlier reports from this laboratory demonstrated that the homogeneous monkey liver serine hydroxymethyltransferase isolated by omitting a heat denaturation step, used by earlier workers (Nakano *et al.*, 1968; Fujioka, 1969; Akhtar & El-Obeid, 1972; Cheng & Haslam, 1972; Rowe & Lewis, 1973; Palekar *et al.*, 1973; Jones & Priest, 1976; Ulevitch & Kallen, 1977) had a molecular weight of 208,000

Abbreviations used: c.d., circular dichroism; SDS, sodium dodecyl sulphate; u.v., ultraviolet; GdmCl, guanidine hydrochloride; $(\theta)_{m.r.w.}$, mean residue ellipticity; H_4 folate., (±)-L-tetrahydrofolate.

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with four identical subunits (Ramesh & Appaji Rao, 1980b) and exhibited both homotropic and heterotropic allosteric interactions (Ramesh & Appaji Rao, 1978). It was also demonstrated that the interaction of Cibacron Blue 3G-A with the monkey liver enzyme (Ramesh & Appaji Rao, 1980a) and also the binding of antibody to it (Ramesh, 1980) produced conformational changes in the enzyme. These observations and the lack of any information on the solution conformation (*i.e.*, the secondary structure) of this protein, prompted the present investigation on the structure-activity relationship of the enzyme.

The present paper reports the results of studies on the changes in the conformation and activity of the monkey liver serine hydroxymethyltransferase on the addition of urea, guanidinium hydrochloride (GdmCl), sodium dodecyl sulphate (SDS), haloalcohols, acid and alkali, using circular dichroic (c.d.) and fluorescence probes.

Materials and methods

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA: 2-mercaptoethanol, pyridoxal 5′-phosphate, EDTA and SDS. DL-[3−^{14}C] serine (48.5 mCi/mmol) was obtained from New England Nuclear (Boston, Massachussets, USA). H_4 folate was generously given by Dr. J. H. Mangum (Brigham Young University, Provo, Utah, USA). GdmCl (ultra pure grade) and urea (recrystallized) were obtained from Schwarz/Mann Research Laboratories, Orangeburg, New York, USA). Trifluoroethanol was purchased from Eastman Kodak Co., Rochester, New York, USA. 2-Chloroethanol was a product of British Drug House Chemicals Ltd., Poole, England. Millipore filters (type HA 0.45 µm, Millipore Corporation, Massachussets, USA) were kindly given by Dr. A. Ashok Kumar (University of Cincinnati Medical Centre, Cincinnatti, Ohio, USA).

Methods

Serine hydroxymethyltransferase from monkey liver was purified as described earlier (Ramesh, 1979; Ramesh and Appaji Rao, 1980b). The enzyme was assayed by a radio-chemical method (Taylor and Weissbach, 1965) using DL-[3−^{14}C] serine as the substrate; the amount of [^{14}C] formaldehyde formed in 15 min was estimated (Ramesh and Appaji Rao, 1978). One unit of enzyme activity was defined as the amount that catalysed the formation of 1 µmol of formaldehyde/min at 37°C at pH 7.4. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

GdmCl was purified by the method described by Nozaki (1972) and urea by repeated crystallization from absolute ethanol. Trifluoroethanol and 2-chloroethanol were distilled before use.

C.d. measurements were performed at 25°C on a Jasco-20 scanning spectropolarimeter. The circular dichrometer was calibrated using an aqueous solution of d-10-camphor sulphonic acid (Cassim and Yang, 1969). All buffers, reagents and protein solutions were passed through Millipore filters before recording the c.d. spectra. The spectropolarimeter was continuously purged with prepurified nitrogen before and during the experiments. All the c.d. spectra were recorded with the slit programmed for 1 nm-spectral band width, a chart speed of 4 nm/min, a time constant of 64s and a wavelength expansion of 5 nm/cm. All c.d. spectral data are plotted as mean residue ellipticity which was calculated using the equation,

$$[\theta]_{m.r.w} = (\theta \times m.r.w.)/(10 \times l \times c)$$

where θ is the observed ellipticity in degrees, m.r.w. is the mean residue weight which is assumed to be 115, l is the optical path in cm and c is the concentration of the enzyme in g/ml.

Fluorescence measurements were made using a Perkin-Elmer model 203 fluorescence spectrophotometer equipped with a 150-W Xenon lamp source. The instrument was standardized using quinine sulphate solution. The emission spectra reported are uncorrected. All readings were made at room temperature, 26 ± 1°C. Quartz cuvettes (1 cm path length) containing 3 ml of enzyme solution were used in the experiments. The enzyme was extensively dialysed against 50 mM-potassium phosphate buffer, pH 7.4 and filtered through a Millipore filter before fluorescence spectral measurements were made. The absorbance of the enzyme at 280 nm was always maintained below 0.10. Appropriate corrections were applied where the total volume change was significant. Fluorescence spectral measurements were made against appropriate controls.

Results

Circular dichroism of monkey liver serine hydroxymethyltransferase

In the near-u.v. c.d. spectral region, the monkey liver enzyme showed negative ellipticity maxima at 280 nm and 270 nm and a shoulder at 276 nm (figure 1a). The spectrum was characterized by a fine structure in the region of absorbance of aromatic amino acids. Both tyrosine and tryptophan residues contribute to the c.d. band at 276 nm (Beychok, 1966) and the 280 nm band may be due to tryptophan transitions (Beychok, 1967).

In the far-u.v. c.d. spectral region (200-250 nm), the c.d. spectrum of the native enzyme was qualitatively that of a protein with α-helical conformation (figure 1b). The spectrum is characterized by two negative ellipticity bands centred at 220-222 nm and 207-209 nm with amplitudes of $(-9.82 \pm 0.11) \times 10^3$ and $(-9.91 \pm 0.10) \times 10^3$, deg. cm^2. dmol^{-1}, respectively. The characteristic c.d. spectrum of the enzyme was not sensitive to protein concentration (0.1 to 3 mg/ml) and to the ionic strength of the solvent (0.03 to 0.2 M). Estimation of the α-helical content from the far-u.v. c.d. spectral data was obtained both by the method of (i) Chen and Yang (1971), using the mean residue ellipticity value at 222 nm and (ii) Greenfield and Fasman (1969), using the mean residue ellipticity at 208 nm. Estimates of the α-helical content by the two procedures gave values of 20 ±0.3% by the method of Greenfield and Fasman (1969) and 25 ±0.25% when the analysis was carried out according to Chen and Yang (1971). Since the contributions of α-helix to the overall secondary structure of the protein, calculated by different methods vary

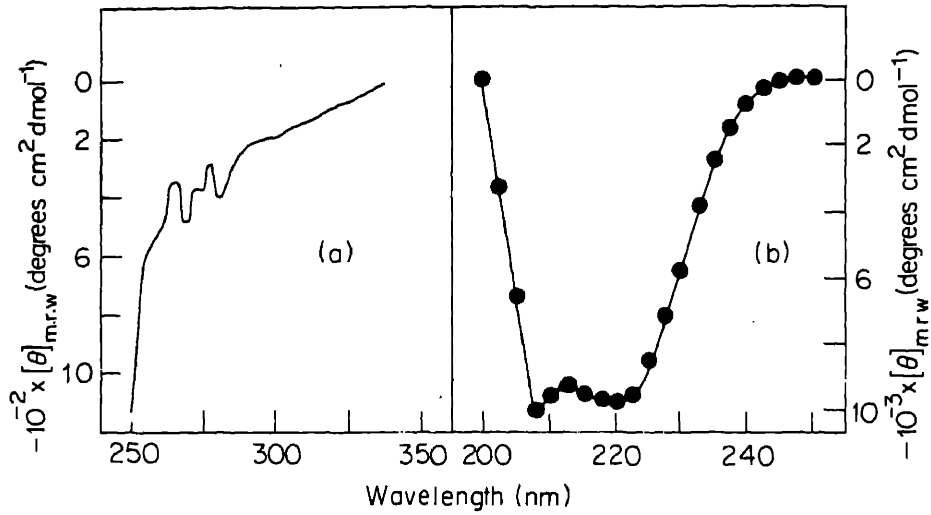

Figure 1. Near- and far-u.v. circular dichroic spectra of monkey liver serine hydroxymethyltransferase. a. The near-u.v. c.d. spectrum of the enzyme (2 mg/ml) in 50mM-potassium phosphate buffer, pH 7.4 containing 1 mM 2-mercaptoethanol and 1 mM EDTA, was recorded in a cell of 1 cm-pathlength containing 3 ml of the enzyme solution. b. The far-u.v. c.d. spectrum of the enzyme (3.28 mg/ml) in 50 mM potassium phosphate buffer, pH 7.4/1 mM 2-mercaptoethanol/1 mM EDTA was recorded by using a demountable quartz cell of 0.1 mm light path. (figure 1b).

significantly (Lee *et al.*, 1978; Luer & Wong, 1978), we have used $-[\theta]_{222}$ values to describe the conformational changes in the enzyme, as it is a sensitive probe of the polypeptide backbone conformation (Leavis *et al.*, 1978).

Effect of urea and GdmCl on the circular dichroic and fluorescence spectra of monkey liver serine hydroxymethyltransferase

The far-u.v. c.d. spectra of the enzyme in the presence of different fixed concentrations of GdmCl are shown in figure 2a. At 0.4 M GdmCl, there was a shift of the negative 221 nm maximum to 217 nm, and at 1.6 M GdmCl, the negative band shifted further to 216 nm. At 6.4 M GdmCl, the c.d. spectrum of the enzyme assumed a pattern similar to that of a randomly coiled polypeptide chain (Stevens *et al.*, 1968) with no characteristic c.d. bands at 221 nm and 208 nm.

The effect of increasing concentrations of urea on the c.d. spectrum of the enzyme in the far-u.v. region is depicted in figure 2b. As is evident from the figure, the presence of 0.8 M urea did not alter the position of the negative ellipticity bands (209 nm and 221 nm), although there was a slight decrease in the peak amplitudes. However, the presence of higher concentration of urea (>1.0M) resulted in a shift of the 221 nm negative ellipticity band to 216 nm. In the presence of 8 M urea, the far-u.v. region c.d. spectrum indicated extensive denaturation.

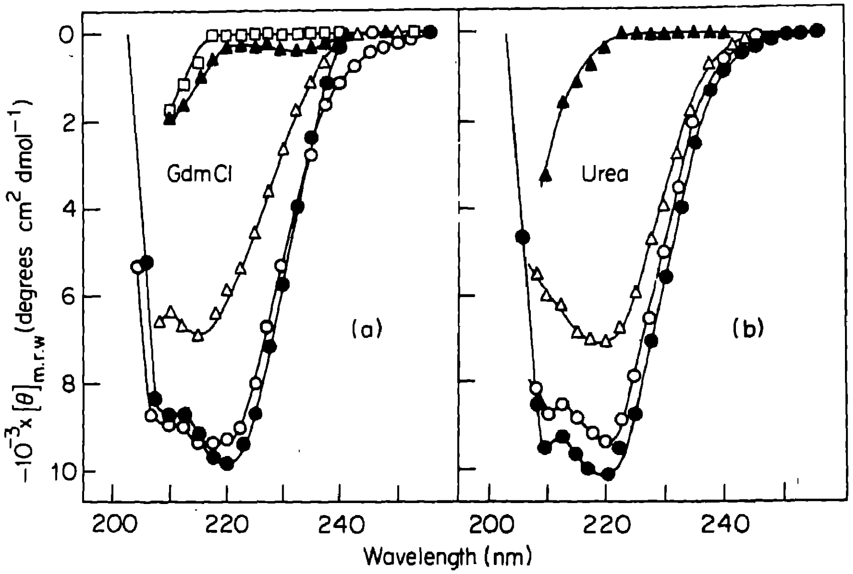

Figure 2. The far-u.v. circular dichroic spectra of monkey liver serine hydroxymethyltransferase as a function of GdmCl and urea. The enzyme 50 μl (3.50 mg/ml) was mixed with 200 μl of urea or GdmCl (concentrations ranging between 0.5 to 10 M) to yield the denaturant concentration indicated below. The c.d. spectra were recorded 1h after mixing the enzyme with the denaturants using 0.5 mm pathlength demountable quartz cell. a. GdmCl, OM (●); 0.4 M (O); 1.6 M (Δ); 4.8 M (▲) and 6.4 M (□). b. Urea, 0 M (●); 0.8 (O); 1.6 M (Δ) and 8 M (▲).

The enzyme exhibited fluorescence emission maxima at 335 nm on excitation at 285 nm and at 340 nm on excitation at 295 nm. The addition of 6 M GdmCl and 8 M urea to the enzyme resulted in a red-shift of the 340 nm-fluorescence emission peak to 365 nm and 350 nm, respectively. This could be attributed to the exposure of tryptophan residues to the solvent or their shift into a more hydrophobic environment and may in turn reflect an altered three dimensional conformation of the enzyme induced by these denaturants (data not shown).

Effect of urea and GdmCl on the enzyme activity and mean residue ellipticity at 222 nm

GdmCl and urea, both inactivated the serine hydroxymethyltransferase and the enzyme inactivation was dependent on the concentration of the denaturant. The enzyme underwent a conformational transition as indicated by the decrease of $-[\theta]_{222}$ values, when the GdmCl concentration was increased from 0 to 5 M, with the mid point of transition at 1.9 M GdmCl. On the other hand, when urea was used as the denaturant, the mid point of transition from native to the denatured state occurred at 3.6 M urea (data not shown). In the case of GdmCl, the loss of enzyme activity was rapid and 50% activity was lost at only 0.4 M GdmCl. In contrast, 2.4 M urea was needed to effect a 50% loss of enzyme activity. The loss of activity and secondary structure of the enzyme was completed within 15 min o exposure to the denaturants. Removal of the denaturants from the fully denatured enzyme solution, by dialysis against potassium phosphate buffer (50 mM/pH 7.4) did not result in the recovery of either the enzyme activity or the secondary structure.

Effect of trifluoroethanol and 2-chloroethanol on the far-u.v. region c.d. spectra and activity of the monkey liver serine hydroxymethyltransferase

The effect of 50 to 90 vol.-% trifluoroethanol and 10-35 vol.-% 2-chloroethanol on the conformation of the enzyme was tested. It was found that 50-90 vol.-% trifluoroethanol altered the conformation significantly (figure 3a) as indicated by an increase in the mean residue ellipticity value of the native enzyme form −10120 at 222 nm to −15870 at 50 vol.-% trifluoroethanol and to −25530 at 90 vol.-% trifluoroethanol; the corresponding values at 208 nm were −10810, −14720 and −30130 deg. cm^2. dmol^{-1}, respectively. Interestingly, the negative ellipticity at 208 nm in the presence of 90 vol.-% trifluoroethanol was about 13% greater than at 222 nm.

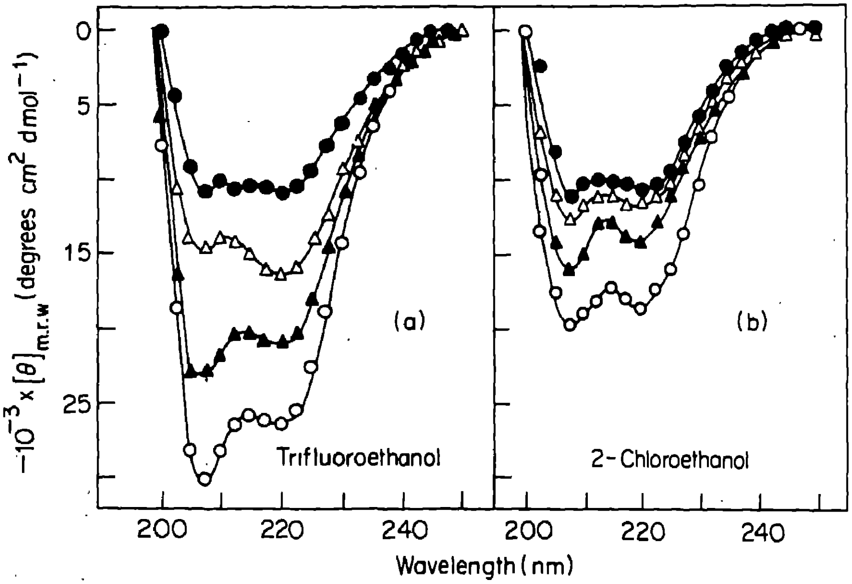

Figure 3. Effect of trifluoroethanol and 2-chloroethanol on the far-u.v. circular dichroic spectra of monkey liver serine hydroxymethyltransferase. The enzyme (0.2 mg/ml) in 10 mM potassium phosphate buffer, pH 7.4/1 mM 2 mcercaptoethanol/1 mM EDTA, was mixed with either trifluoroethanol or 2-chloroethanol to obtain a final concentration indicated in figure. The c.d. spectra were recorded after 1h of equilibration using a demountable quartz cell of 0.5 mm-light path. Trifluoroethanol below 50 vol.-% precipitated the protein and 2-chloroethanol above 35 vol.% failed to increase the $-[\theta]_{m.r.w.}$ values. a. Trifluoroethanol, 0% (●); 50 vol.-% (Δ) 60 vol.-% (▲) and 90 vol.-% (O). b. 2-Chloroethanol, 0% (●); 10 vol.-% 20 vol.-% (▲) and 35 vol.-% (O).

2-Chloroethanol was more effective than trifluoroethanol in increasing the mean residue ellipticity values (figure 3b). Thus, 90 vol.-% trifluoroethanol was required to increase the $-[\theta]_{222}$ values from -10120 to -25530 deg. cm^2.dmol^{-1}, whereas, only 35 vol.-% 2-chloroethanol was required to increase the $-[\theta]_{222}$ values from -10120 to -17420 deg. cm^2. dmol^{-1}. The enzyme activity was completely lost on the addition of very low concentration (1%) of these alcohols. Extensive dialysis of the enzyme-alcohol mixtures did not result in the restoration of the native enzyme structure, as indicated by $-[\theta]_{222}$ values and enzyme activity, although a small decrease (10%) in the $-[\theta]_{222}$ value was observed with trifluoroethanol.

Influence of SDS on the circular dichroism and fluorescence spectrum of monkey liver serine hydroxymethyltransferase

The addition of 0.4%-SDS to the enzyme altered the native conformation to a more ordered structure. Inspection of figure 4 indicates the absence of an explicit 222 nm negative ellipticity band and the prevalence of a plateau at 208-222 nm. The enzyme was completely inactive in the presence of low concentrations of SDS (0.01%) and this process was irreversible, as monitored by activity and conformation.

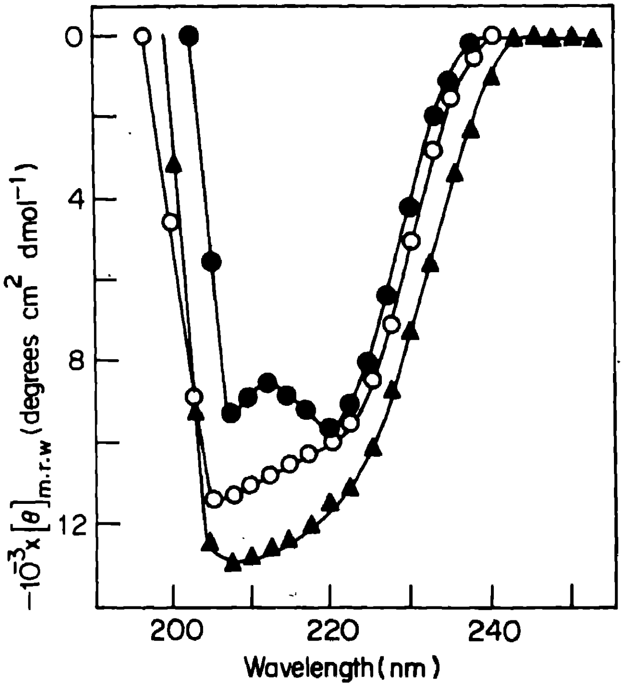

Figure 4. Far-u.v. circular dichroic spectra of monkey liver serine hydroxymethyltransferase in the presence of SDS. The enzyme in 50mM potassium phosphate buffer, pH 7.4 was exhaustively dialysed against 50 mM sodium phosphate buffer, pH 7.4, before mixing with SDS, in order to avoid the precipitation of SDS. The enzyme (0.18 mg/ml) was mixed with SDS and the c.d. spectra were recorded after 1h of equilibration at 25°C using a 0.5 mm-path length demountable quartz cell. 0% SDS (●); 0.2% (○) and 0.4% (▲).

Interaction of SDS with the enzyme resulted both in increased fluorescence intensity as well as a red-shift of the emission maximum to 360 nm.

pH-Induced alterations in the secondary structure and activity

The far-u.v. c.d. spectra of the enzyme at pH 1.4, 7.4 and 12.6 are shown in figure 5. The c.d. spectrum of the enzyme at pH 1.4 differed considerably from that at pH 7.4. There was an overall decrease in the c.d. band intensities at 222 nm and 208 nm. The 222 nm band shifted to a lower wavelength and these changes were consistent with a gradual loosening of the secondary structure. Even more drastic changes in the circular dichroic characteristics were observed at alkaline pH. At pH 12.6, the spectrum of the enzyme assumed the character of a denatured, but still constrained protein (Fasman *et al.*, 1970), with a negative maximum at 205 nm and a broad shoulder at 220 nm.

The changes in the negative ellipticity at 222 nm and the activity as a function of pH are shown as an inset to figure 5. The magnitude of the 222 nm negative ellipticity band increased significantly from pH 1.4 to 7.5 and was followed by a more gradual decrease above pH 7.6. The pH-activity profile also followed a similar

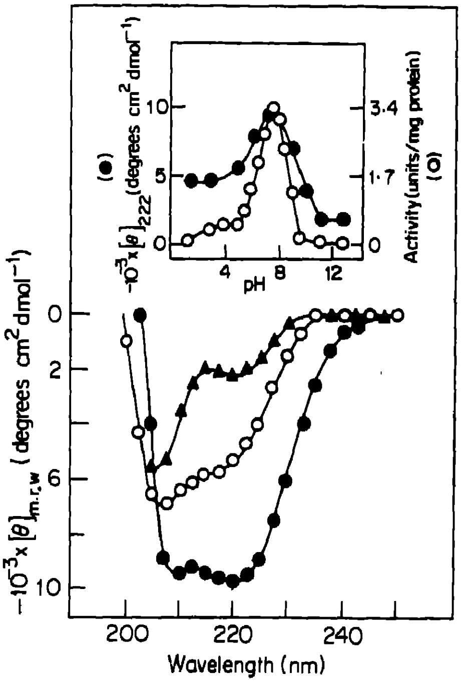

Figure 5. Alteration in the far-u.v. circular dichroic spectrum and the enzymic activity of serine hydroxymethyltransferase as a function of pH.

The enzyme (0.3 mg/ml) in 20 mM potassium phosphate buffer, pH 7.4, was adjusted with either 0.2 M HCl or 0.2 M NaOH to the desired pH value and the c.d. spectra recorded after 30 min equilibration at 25°C, using a 0.5 mm-light path demountable quartz cell. pH 12.6 (▲); pH 1.4 (O) and pH 7.4 (●). The inset shows the changes in mean residue ellipticity at 222 nm (●) and enzyme activity (O) as a function of pH. The activity of the enzyme was assayed by the procedure described in Methods.

pattern, the enzyme exhibiting maximal activity at pH 7.45. It was interesting to note that the enzyme had lost most of its activity between pH 1.5 and 4.0, although the negative ellipticity values at 222 nm testified the prevalence of residual structural constrains in the enzyme. The acid-transition was reversible above pH 5.0, but was irreversible at lower pH values. The loss in the secondary structure and activity of the enzyme above pH 9.8 could not be regained by readjusting the pH to neutral values.

Discussion

The elucidation of the nature of the forces that stabilize the native conformation of proteins and the steps in the pathways of protein unfolding and refolding, have been a major focus of protein research in the last few decades. A very versatile approach for the understanding of the conformation of proteins in solution is the study of protein denaturation. The results of the investigation described in this paper represent a preliminary attempt to demonstrate the usefulness of using intrinsic fluorescence and circular dichroism as probes to examine the conformational changes induced by interactions with various denaturants and enzyme activity.

The near u.v. c.d. spectrum of the monkey liver serine hydroxymethyltransferase (figure 1a) appears unusual in the absence of positive c.d. signals. A similar type of spectrum has been reported in the case of *E. coli* ribosomal protein L7/L12 (Luer and Wong, 1979). The absorption spectrum of the enzyme (figure 5, Ramesh & Appaji Rao, 1980b) shows a peak at 420 nm, due to the presence of protein bound pyridoxal 5′-phosphate. The presence of bound pyridoxal 5′-phosphate could be one of the reasons for this rather unusual c.d. spectrum.

The far-u.v. c.d. spectrum of the enzyme (figure 1b) was typical of a protein which contained an unique conformation with a well organized secondary structure. The c.d. spectrum of the enzyme in 6.4 M GdmCl and 8M urea (figure 2) was characteristic of a protein which was unfolded into an extremely disordered conformation (Nozaki *et al.*, 1974). There was a good correlation between the loss of native structure (as monitored by $-[\theta]_{222}$ values) and loss of enzyme activity, when urea was used as the denaturant; whereas, in the case of GdmCl, the enzyme activity was lost much earlier compared to the loss of secondary structure. These results suggested that GdmCl might initiate conformational changes close to or at the active site; whereas, urea might initiate denaturation at a site distal to the substrate binding site of the enzyme. It was also noted that higher concentrations of urea were required to effect complete unfolding as compared to GdmCl and this might be due to the differences in the structure of the two denaturants. It was interesting to observe that the final c.d. spectra of the enzyme in 6.4 M GdmCl or 8 M urea were nearly similar. This would suggest that the pathways of protein unfolding induced by GdmCl and urea might be different, although the resulting denatured products were similar (Hibbard and Tulinsky, 1978). The fact that the GdmCl-or urea-denatured enzyme, failed to regain either the catalytic activity or the native structure, would imply that the process of denaturation was essentially irreversible. This is not surprising, since, in oligomeric proteins, the reassociation of individual subunits to form the native quaternary structure plays an important role in the overall renaturation process.

At a high concentration of trifluoroethanol or 2-chloroethanol, the enzyme showed a transition from the native structure to a conformation with a considerable proportion of α-helix (figure 3) as indicated by enhanced mean residue ellipticity values at 208 nm and 222 nm. The effects of ethanol and similar water-miscible solvents on protein conformation have been discussed by several investigators (Tanford, 1968; Timasheff, 1970; Jirgensons, 1978; Toniolo *et al.*, 1978; Tamura and Jirgensons, 1980). Jirgensons (1978) reported that non-helical proteins (*e.g.*, β-lactoglobulins and caseins) were reorganized into new conformations of higher helical content than in the native state. Our results of the helix promoting effects of trifluoroethanol and 2-chloroethanol on the structure of the oligomeric serine hydroxymethyltransferase appears to be an unique example of a large mol.wt. protein undergoing these transitions. The fact that the activity of the enzyme was lost in the presence of the haloalcohols, suggested that the active site geometry and its integrity might have been lost during the interactions.

Similar to alcohols, the interaction of SDS with serine hydroxymethyltransferase resulted in the enzyme adopting a new conformation with greater helical content than in the native state (figure 4). Jirgensons (1976) proposed that SDS-induced conformational transitions occurred in two stages: (i) interaction in the first stage resulted in the unfolding of the ordered globular proteins and (ii) the unfolded polypeptide chain refolded into another conformation of higher order, which differed from the native conformation. The loss of enzymic activity in the presence of SDS might be due to the altered structure of the enzyme in the presence of the denaturant. Although a number of models have been put forward to explain the varied hydrodynamic behaviour of protein-SDS complexes, which contain approximately 1.4g SDS/g of protein (Reynolds and Tanford, 1970; Shirahama *et al.*, 1974; Wright *et al.*, 1975; Mattice *et al.*, 1976) none of these models provide a molecular explanation for their formation.

Significant alterations in the c.d. spectra of the enzyme were induced by pH alterations (figure 5) suggesting an unfolding of the native conformation. A permanent loss of the secondary structure of the enzyme occurred below pH 4.0, since the spectrum obtained at pH 4.0 remained irreversibly distorted upon restoration to neutral pH values. At extremes of pH, the c.d. spectrum of the enzyme became increasingly dominated by a disordered structure and resembled the spectrum of a randomly coiled protein and these processes were irreversible. The enzyme was not dissociated into subunits over the entire pH range as monitored by isoelectrofocusing and polyacrylamide gel electrophoresis (Ramesh, 1980). The interpretation of the c.d. spectra of the enzyme at alkaline pH values was complicated due to the increased reactivity of —SH groups at high pH values and peptide bond cleavage (Lapanje, 1978). The good correlation observed between negative ellipticity values at 222 nm and enzymic activity at different pH values (inset to figure 5), suggested that, for the enzyme to be optimally active, its structure should exist in a precise conformation. Thus at pH 7.4, the enzyme had maximal activity as well as $-[\theta]_{222}$ value.

In conclusion, the solution coformation of the monkey liver serine hydroxymethyltransferase reported for the first time, possesses a well defined secondary structure, which when perturbed by denaturants or pH alterations, produces changes both in the structure and the activity of the enzyme. Our results establish that this enzyme offers a useful handle for understanding the mechanism of protein denaturation.

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Allosteric serine hydroxymethyltransferase from monkey liver: temperature induced conformational transitions

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Abstract. The homogeneous serine hydroxymethyltransferase from monkey liver was optimally activate at 60°C and the Arrhenius plot for the enzyme was nonlinear with a break at 15°C. The monkey liver enzyme showed high thermal stability of 62°C, as monitored by circular dichroism at 222 nm, absorbance at 280 nm and enzyme activity. The enzyme exhibited a sharp co-operative thermal transition in the range of 50°-70° (T_m=65°C), as monitored by circular dichroism. L-Serine protected the enzyme against both thermal inactivation and thermal disruption of the secondary structure. The homotropic interactions of tetrahydrofolate with the enzyme was abolished at high temperatures (at 70°C, the Hill coefficient value was 1.0). A plot of *h* values vs. assay temperature of tetrahydrofolate saturation experiments, showed the presence of an intermediate conformer with an *h* value of 1.7 in the temperature range of 45°-60°C. Inclusion of a heat denaturation step in the scheme employed for the purification of serine hydroxymethyltransferase resulted in the loss of co-operative interactions with tetrahydrofolate. The temperature effects on the serine hydroxymethyltransferase, reported for the first time, lead to a better understanding of the heat induced alterations in conformation and activity for this oligomeric protein.

Keywords. Serine hydroxymethyltransferase; circular dichroism; thermal stability; conformational transitions.

Introduction

The kinetic, regulatory, physicochemical, immunological studies (Ramesh, 1979, 1980; Ramesh and Appaji Rao, 1978, 1980a, 1980b) and the investigation on the denaturation (Ramesh *et al.*, 1980), suggested that the oligomeric serine hydroxymethyltransferase from monkey liver exhibited conformational fluctuations consequent to the binding of effectors, ligands and change in the solution environment. It was quite surprising to note that the temperature-dependence of the reaction

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Abbreviations used: c.d., circular dichroism; u.v., ultraviolet; $[\theta]_{m.r.w.}$, mean residue ellipticity; H_4folate, (+)-L-tetrahydrofolate; dimedone, 5,5-dimethylcyclohexane-1,3-dione.

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catalyzed by serine hydroxymethyltransferase from any source had not been reported, although heat inactivation of contaminating proteins in the presence of L-serine has been used as a major step in the purification of this enzyme from several sources (Nakano *et al.*, 1980; Fujioka, 1969; Rowe and Lewis, 1973; Jones and Priest, 1976). An earlier report from this laboratory showed that this enzyme from monkey liver isolated without the heat-denaturation step, exhibited both homotropic and heterotropic co-operative interactions (Ramesh and Appaji Rao 1980b). It was therefore of interest to examine the nature of the temperature-induced effects on the protein. These temperature-induced transitions, monitored using spectroscopic and kinetic methods, are described in this paper.

Materials and methods

Materials

DL-[3−^{14}C] Serine (specific activity 48.5 mCi/mmol) was obtained from New England Nuclear (Boston, Massachussets, USA). H_4folate prepared by the method of Hatefi *et al.* (1960), was generously given by Dr. John H. Mangum (Brigham Young University, Provo, Utah, USA). Pyridoxal 5′-phosphate, EDTA, and 2-mercaptoethanol were products of Sigma Chemical Co. (St. Louis, Missouri, USA).

Methods

Serine hydroxymethyltransferase from monkey liver was purified as described earlier (Ramesh, 1979; Ramesh and Appaji Rao, 1980b). The enzyme was assayed by a radiochemical method (Taylor and Weissbach, 1965) using DL-[3−^{14}C] serine as the substrate; and the amount of [^{14}C] formaldehyde formed in 15 min was estimated (Ramesh and Appaji Rao, 1978). One unit of enzyme activity was defined as the amount that catalyzed the formation of 1 μmol of formaldehyde/min at 37°C, pH 7.4. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Circular dichroic measurements were performed on a Jasco-20 scanning spectropolarimeter. The circular dichrometer was calibrated using an aqueous solution of d-10-camphor sulphonic acid (Cassim and Young, 1969). All buffers, reagents and enzyme solutions were passed through Millipore filters (HA 0.45 μm) before recording the c.d. spectra. The spectropolarimeter was continuously purged with pre-purified nitrogen before and during the experiments. All the c.d. spectra were recorded with the slit programmed for 1 nm-spectral band width, a chart speed of 4 nm/min, a time constant of 64 s and a wavelength expansion of 5 nm/cm.

Temperature effects were studied in jacketed cells through which an ethylene glycol-water mixture was circulated from a thermostatically controlled water circular bath. The temperature of the sample was increased in a stepwise manner at a constant rate. Temperature was measured in the cell holder by making use of the mercury contact. A quartz cell of 1 nm-pathlength was used in melting-profile experiments. All c.d. spectral data were plotted as mean residue ellipticity θ, and was calculated using the formula:

$$[0]_{m.r.w.} = (\theta \times m.r.w.)/(10 \times l \times c),$$

and expressed in units of degree cm^2.dmol^{-1}; where 0 is the observed ellipticity in degrees, m.r.w. is the mean residue weight (assumed to be 115), *l* is the optical pathlength in cm and *c* is the concentration of the enzyme in g/ml.

Results

Effect of temperature on the velocity of the reaction catalyzed by monkey liver serine hydroxymethyltransferase

Arrhenius plot of log *v* (velocity at saturating concentration of substrates) vs. the reciprocal of absolute temperature (*T*) and a plot of *v* vs. the temperature (°C) are shown in figure 1. The inset plot shows 60°C as the optimal temperature for the

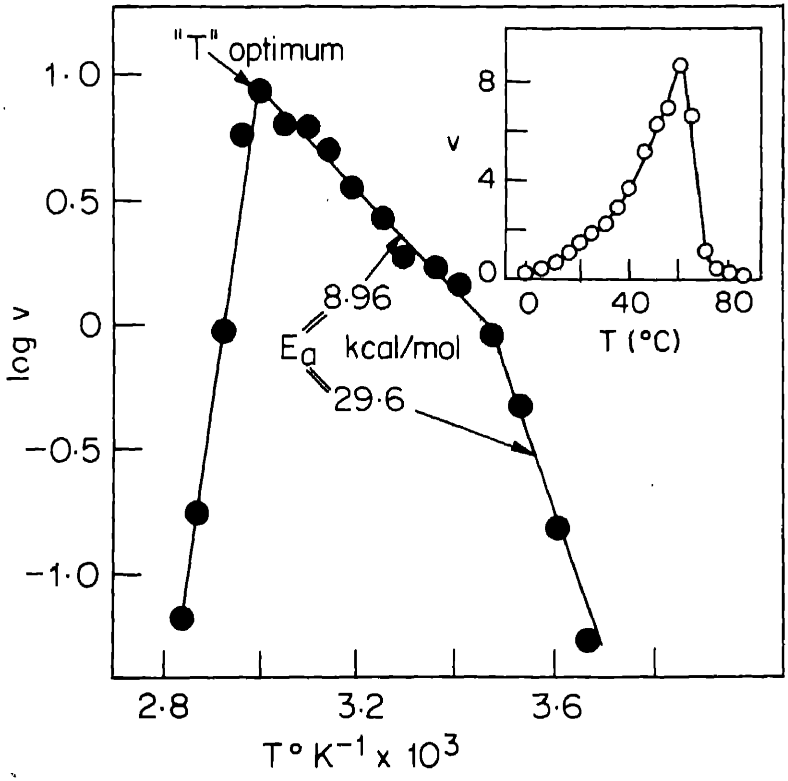

Figure 1. Arrhenius plot for the monkey liver serine hydroxymethyltransferase. The activity of the enzyme (1.1 μg in 50 mM potassium phosphate buffer, pH 7.4) was assayed at different fixed temperature (0-80°C) in the presence of saturating concentrations of L-serine (3.6 mM) and H_4folate (1.8 mM). The H_4fclate concentration was increased to 2.1 mM, when the enzyme activity was measured above 50°C. This was to ensure that saturating concentrations of the substrates were present at all temperatures. The inset shows the plot of initial velocity vs. temperature of the assay in degrees centigrade.

reaction catalyzed by serine hydroxymethyltransferase. The Arrhenius plo showed a linear increase between 0°C to 60°C with a break at 15°C. Activation energies (E_a) calculated from the slopes in figure 1, were 29.6 K cal mol^{-1} (0-15°C) and 9 K cal mol^{-1} (15-60°C). The velocity decreased rapidly at higher temperatures (> 62°C) due to inactivation of the enzyme.

Thermal stability

The thermal stability was monitored by measuring enzyme activity, the absorbance at 280 nm and the c.d. spectrum in the region, 200-250 nm. In these experiments, the enzyme was heated at different temperatures and for different time intervals and quickly cooled to 0°C. The enzyme activity and the spectral properties were measured at 37°C and 25°C, respectively.

It is evident from figure 2 that the enzyme was stable at 50°C for 20 min. At 65°C, there was a progressive loss of enzyme activity with increasing periods of heating. However, at 70°C, the enzyme was rapidly inactivated, with complete loss of activity occurring in 6 min. Similar denaturation profiles were obtained when the enzyme concentration was increased from 100 μg to 1 mg/400 μl buffer; suggesting that the rate of thermal inactivation was not dependent on the concentration of the enzyme.

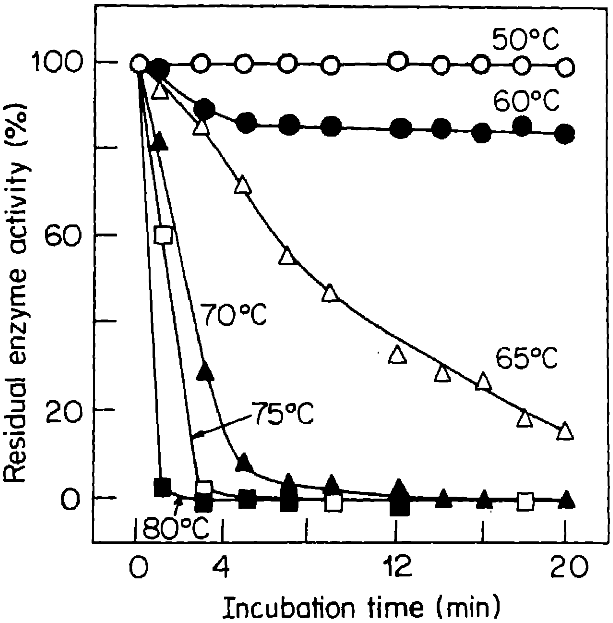

Figure 2. Thermal stability: Effect of pre-incubation temperature on the enzyme activity. The enzyme (100 μg/400 μl of 50 mM-potassium phosphate buffer, pH 7.4) was heated at different temperatures (50°-80°C) and aliquots were withdrawn at the time intervals indicated in the figure. The aliquots were cooled in ice. All the samples were warmed to 37°C by incubating for 5 min in a water-bath and the enzyme assayed at 37°C. The activity of a control sample maintained at 37°C was normalized to 100. The results of the experiment are expressed as per cent of the activity of unheated controls.

Figure 3 shows the family of far-u.v. c.d. spectral curves observed on increasing the temperature from 25° to 70°C. The prominent features of the helix related-c.d. spectrum remained unperturbed upto 50°C. Above 50°C, the amplitudes of the c.d. spectral bands decreased and at 67°C, the spectrum observed was consistent with that of a largely unfolded polypeptide chain.

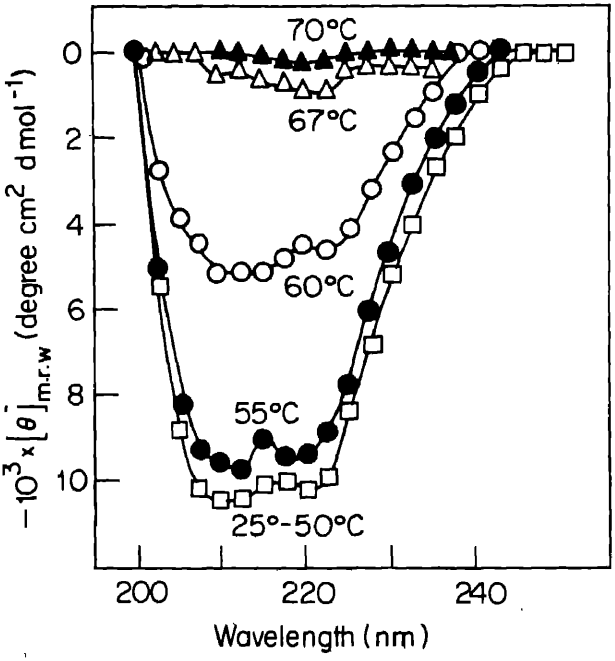

Figure 3. Thermal stability: Effect of pre-incubation temperature on the far u.v. circular dichroic spectra of the enzyme. The enzyme (270 μg/ml) was heated at different temperature (25°-70°C) for 10 min and immediately chilled in ice. C.d. spectra were recorded at 25°C using a demountable quartz cell of 0.5 mm-light path. Above 65°C, the enzyme solution became slightly turbid and these samples were centrifuged before recording the c.d. spectra.

Figure 4 summarizes the relationship obtained between (i) $-[\theta]_{222}$, a sensitive parameter of polypeptide backbone conformation, (ii) A_{280}, a parameter related to tertiary structure of the protein, and (iii) enzyme activity, a parameter related to structural integrity of the active site, and the preincubation temperature. As can be seen from the figure, there was no decrease of either the activity or the $[\theta]_{222}$ or A_{280} values upto 52°C. Above this temperature, there was a sharp and parallel decrease in these parameters indicative of a major conformational change. The mid-points of the unfolding transition calculated from these curves are around 62°C.

Thermal melting profile

The melting-profile of the enzyme was determined by continuously monitoring the c.d. signal at 222 nm in the temperatue range of 10°-85°C. Figure 5 shows the temperature dependence of the mean residue ellipticity, $[\theta]$ at 222 nm, with a sharp

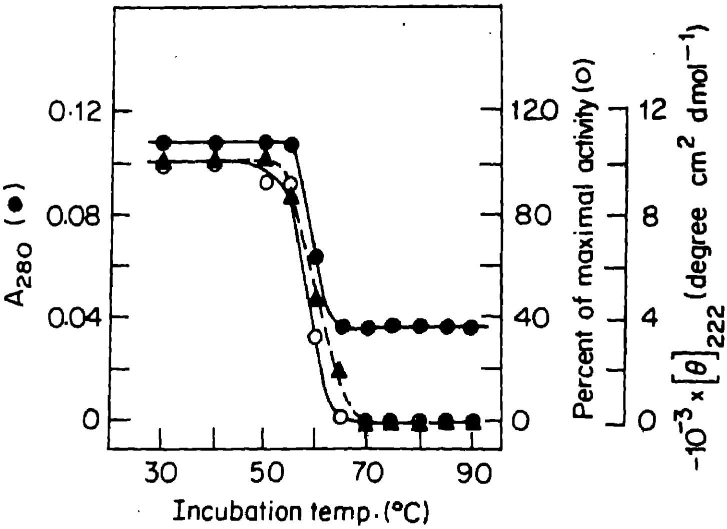

Figure 4. Effect of preincubation temperature on the structure (A_{280} and $-[\theta]_{222}$) and enzyme activity. The enzyme (270 µg/ml) was heated at different temperatures (30°-90°C) for 10 min and immediately chilled in ice. The sample was used to measure the $-[\theta]_{222}$ value (Δ), A_{280} (O) at 25°C and enzyme activity at 37°C (O). Activity of the unheated enzyme was normalized to 100.

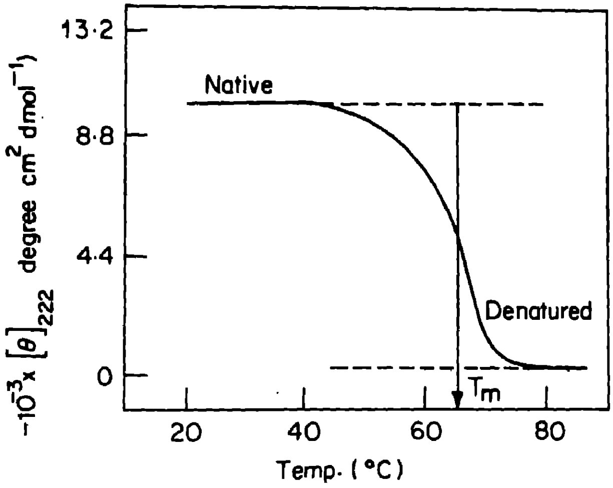

Figure 5. The thermal melting profile. A quartz cell of 1 mm path length and a protein concentration of 0.5mg/ml were used to monitor the c.d. signals at 222nm. It was ascertained that no significant changes in the concentration of the enzyme occurred at high temperatures due to evaporation, by tightly closing the cell with a cap and parafilm. The temperature of the enzyme solution in the cell was gradually increased in a step-wise manner from 20° to 85°C. The circular dichroic signals were recorded after equilibrating the cell for at least 10min, after each desired temperature was attained.

co-operative thermal transition in the range of 50°-70°C. A melting temperature (T_m, defined as the temperature at which the melting-profile crosses the mid-point between the two values extrapolated from the native and the denatured region of the macromolecule) of 65°C was observed. When the enzyme was heated at temperatures above 50°C, cooled and $[\theta]_{222}$ values determined, the values obtained were identical to those observed at the corresponding elevated temperatures.

Effect of substrates on the thermal stability of the enzyme

The susceptibility of the enzyme to thermal inactivation in the absence and in the presence of added substrates is shown in figure 6. In the absence of any substrate, the enzyme lost its activity within 6-7 min on heating at 70°C. However, in the presence of L-serine (18 mM), there was only slight inactivation (<2%) within a 5 min period and at 10 min, 20% inactivation of the enzyme occurred. After this time, there was no further loss of activity upto 20 min. Addition of H_4folate to the enzyme prior to heating at 70°C, did not result in significant protection of the enzyme from inactivation. NADH and NADPH (allosteric activators, upto 10 mM) exerted no significant influence on the thermal inactivation of the enzyme at 70°C.

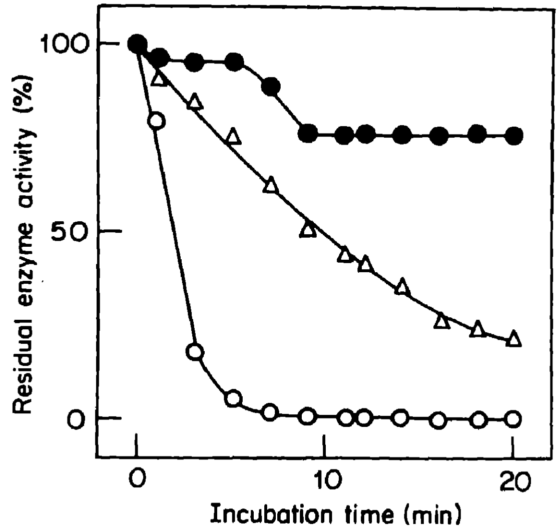

Figure 6. Heat-inactivation patterns of the enzyme in the presence of L-serine and H_4folate The enzyme (100 μg/400 μ/ of 50 mM potassium phosphate buffer, pH 7.4) in the presence of 18 mM L-serine (●), 9 mM H_4folate (Δ) and enzyme alone (O) was heated at 70°C. Aliquots were removed at definite time intervals and the thermal inactivation of the enzyme was terminated by chilling them in ice. The enzyme activity was assayed at 37°C. The enzyme activity of the sample which was not subjected to heat treatment was normalized to 100.

To understand the relationship between the observed structural transition and the catalytic function, circular dichroism was used in order to confirm the presence of conformational transition in the enzyme which exhibited substrate-induced thermal stability (Ramesh and Appaji Rao 1980b). As can be seen in figure 7, the c.d. spectrum of the enzyme heated at 70° C (10 min) in the presence of L-serine (18 mM) showed significant c.d. ellipticitybands at 221 and 208 nm, as compared to the c.d. spectrum of the enzyme obtained on heating at 70° C without the addition of L-serine. The latter spectrum indicated the absence of even the traces of secondary structure in the enzyme. For comparison, the spectrum of the enzyme, which was not subjected to heating at 70°C is shown in figure 7.

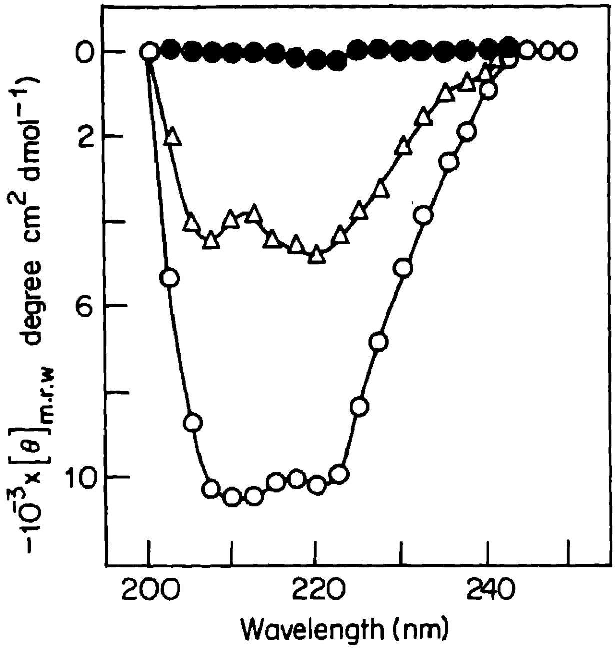

Figure 7. Protective effect of L-serine against temperature-induced loss of circular dichroic properties of the enzyme. The enzyme (270 µg/ml) was heated for 10 min at 70°C in the absence (●) and in the presence (Δ) of 18 mM L-serine. The sample was immediately chilled in ice and the c.d. spectra recorded at 25°C using a 0.5 mm light path demountable quartz cell. For comparison, the spectrum of the enzyme which was not heated at 70°C is shown (O).

Effect of temperature on the positive homotropic interaction of H_4folate (i.e., Hill coefficient, h) with the enzyme

Figure 8 depicts the *h* values plotted vs. incubation temperature. It is evident from the figure that the *h* value of 2.5 was not altered upto 38°C and above this temperature there was a rapid decrease in the *h* value upto 45°C ($h = 1.7$). The *h* value of 1.7 remained constant upto 60°C and above this temperature there was a further decline in the *h* value; at 70°C, the Hill coefficient reached a value of 1.0. The H_4folate saturation curve at 70°C was hyperbolic and the Lineweaver-Burk plot was linear.

The enzyme purified using heat denaturation step (70° C, 5 min) failed to exhibit positive homotropic co-operative interactions with H_4folate. This is indicated by a hyperbolic saturation pattern (figure 9) with a Hill coefficient (*h*) value of 1.1 (inset to figure 9).

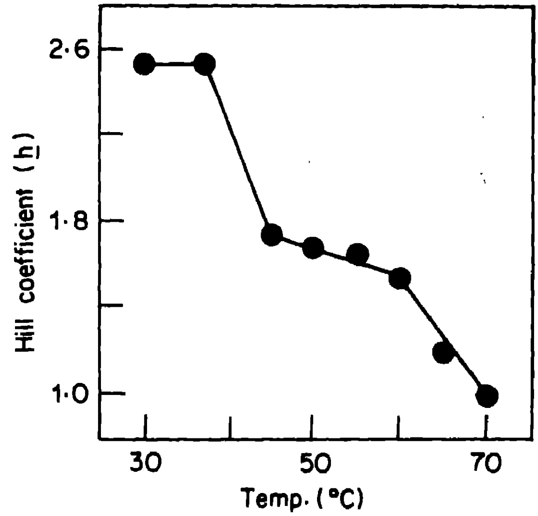

Figure 8.. Effect of temperature on the Hill coefficient of H_4folate saturation of the enzyme The enzyme (1 µg) was preincubated with different concentrations of H_4folate (0.036-2.2 mM) for 5 min and then for 15 min with 3.6 mM L-[3-^{14}C] serine at different fixed temperatures (30°-70°C). After terminating the reaction by the addition of dimedone, the amount of [^{14}C] formaldehyde formed was estimated (Ramesh and Appaji Rao, 1978). The Hill coefficient (*h*) was calculated from a Hill plot of the H_4folate saturation curve at each temperature (Segel, 1975).

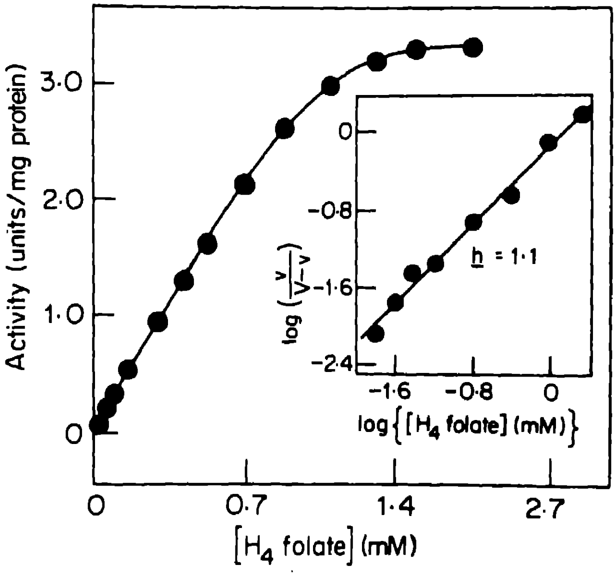

Figure 9. H_4folate saturation pattern of the enzyme purified using heat denaturation step. The enzyme from monkey liver was purified by heating the crude extract for 5 min at 70°C, followed by $(NH_4)_2SO_4$ fractionation, CM-Sephadex chromatography, ultrogel-ACA34 gel filtration and Blue Sepharose CL-6B affinity chromatography (Ramesh & Appaji Rao, 1980b). This enzyme preparation was used for the H_4folate saturation experiment (for details see Ramesh and Appaji Rao, 1978). The inset shows the Hill plot of the data obtained.

Discussion

Thermally induced conformational transitions have been accepted as explanations for the temperature dependence of a number of properties of macromolecules (Massey *et al.*, 1966; Berman and Boyer, 1972; Kelley and Von Hippel, 1976; Zav'yalov *et al.*, 1977; Sturtevant and Matco, 1978; Fujita *et al.*, 1979; Matheson and Scheraga, 1979a, b; Ralston and Dunbar, 1979; Vita *et al.*, 1979 Ugarovo *et al.*, 1979; Luer and Wong, 1980) and have helped in the understanding of the forces that stabilize the higher order structure of proteins and of protein-ligand interactions. The Arrhenius plot for the monkey liver serine hydroxymethyltransferase showed a biphasic curve, with a break at 15°C (figure 1) suggesting temperature-induced conformational change in the molecule. Non-linear Arrhenius plots similar to that observed in the present study have been reported for a few proteins (Sizer, 1943; Levy *et al.*, 1959; Massey *et al.*, 1966; Saint-Blancard *et al.*, 1977; Matheson and Scheraga, 1979b; Berge *et al.*, 1980) and have been interpreted to suggest temperature induced conformational changes. Before invoking this, possibilities such as (i) phase change in the solvent, (ii) failure to maintain saturation of the enzyme with the substrates at elevated temperatures, (iii) artifacts of mixing, (iv) reversible enzyme inactivation, and (v) buffer effects have to be ruled out (Dixon and Webb, 1958). These have been carefully considered and ruled out before invoking conformational change in the enzyme. Thus the nonlinear Arrhenius plot provides evidence in favour of the existence of non-denaturational transitions and also illustrates the concept of limited molecular rearrangement as a possible mechanism for metabolic regulation.

Temperature induced transitions in the enzyme structure are evident from the changes in A_{280} which is indicative of alterations in the tertiary structure; $-[\theta]_{222}$ values, which are correlated to secondary structure of the protein; and the loss of enzyme activity reflecting alterations in the catalytic site (figure 4). By using thermal unfolding as a sensitive probe to measure changes in the different orders of structural organization in the enzyme molecule, it was found that the loss of enzyme activity, and the decrease in A_{280} and $-[\theta]_{222}$ values followed a similar pattern suggesting that these properties are closely related. A similar T_m (62°C) value was obtained, for all the parameters plotted as a function of pre-incubation temperature. A similarity of the melting profiles and the occurrence of an identical T_m value for different conformational probes have been used as evidence for a two-state co-operative unfolding process, without observable intermediates (Lumry *et al.*, 1966).

The changes in $-[\theta]_{222}$ values displayed a sigmoidal dependence on the temperature (figure 5) in the range of 50°-70°C. A T_m value of 65°C was calculated from this curve. It was also seen that in the region of the major thermal transition (50°-70°C), no evidence for intermediates were found. These results are compatible with the hypothesis that two-state co-operative transitions occur during the thermal denaturation of monkey liver serine hydroxymethyltransferase. However, this can be confirmed only after performing precise differential scanning microcalorimetric studies and fast reaction temperature-jump experiments.

The binding of L-serine to the enzyme confers significant thermal stability to the enzyme, whereas H_4folate affords only meagre protection against thermal inactivation (figure 6). These results suggest that the binding of L-serine promotes a conformational change in the native enzyme to a thermostable conformer and this conclusion has been supported by circular dichroism studies (figure 7). Ligand induced stabilization against heat inactivation may also be explained by invoking protection by the substrate of heat labile groups at or near the active site on the enzyme molecule. Similar explanations for substrate protection have been given by several workers (Grisolia and Joyce, 1960; Segel *et al.*, 1968; Theorell and Tatemoto, 1971; Citri, 1973).

At high temperatures, above 65°C, the H_4folate saturation curve became hyperbolic, as indicated by a Hill coefficient value of 1.0 (figure 8). In other words, the homotropic co-operative interactions of H_4folate with the enzyme was abolished at high temperatures. At temperatures above 60°C, the critical interaction between subunits seems to have been lost. These results are further confirmed by desensitization of the enzyme using a heat step during the purification (figure 9). Although the studies on the effect of temperature on the structure of the enzyme clearly indicated the absence of intermediates in the process of denaturation (as monitored by c.d., A_{280}, and enzyme activity), the effect of temperature on the Hill coefficient for the interaction of H_4folate with the enzyme, showed the presence of an intermediate conformer with an h value of 1.7. The mol. wt of the native as well as the temperature desensitized enzyme were identical indicating that dissociation of the enzyme may not have occurred at elevated temperatures (data not given). These results would suggest that intermediate conformations are possible during the process of thermal denaturation. The apparent discrepancy in the results obtained by spectroscopic methods and the kinetic methods, may be due to the differences in the sensitivity of these two probes.

In conclusion, the results presented in this paper suggest that temperature induced conformational changes may have a role in the metabolic regulation of serine hydroxymethyltransferase.

Acknowledgements

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Expression of Hbβ -T and Hbβ -E genes in Eastern India — Family studies

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Abstract The distribution patterns of different haemoglobins were observed amongst the family members of β-thalassaemia homozygous and HbE-β-thalassaemia patients with the aid of gel electrophoretic and alkali denaturation techniques. Of the 18 families studied, four belonged to β-thalassaemia homozygous and 14 to HbE-β-thalassaemia patients. Interaction of HbE and β-thalassaemia genes resulted in major clinical abnormalities with increase in the percentages of haemoglobins F and E. The percentages of HbA_2 in homozygous β-thalassaemia were within the normal range. Although in Southeast Asia the β° type of HbE-thalassaemia is more prevalent, only one individual with this type of thalassaemia was observed during this survey. In the rest of the patients examined the percentages of adult haemoglobin ranged from 5.2 to 42.5 indicating the presence of a β^{+} type gene.

Keywords. β-thalassaemia; haemoglobin E; haemoglobin A

Introduction

Haemoglobin E is found in relatively high frequencies in the eastern part of India and has been suggested to act as a marker for the mongoloid element (Flatz *et al.*, 1972, Das and Flatz, 1975). It occurs both in heterozygous and homozygous forms and frequently in combination with β-thalassaemia. The effect of the genetic interaction between HbT and HbE has been known to result in more drastic clinical expression than β-thalassaemia heterozygote and HbE homo- and heterozygotes (Chatterjea *et al.*, 1956, Chernoff *et al.*, 1956, Lie-Injo, 1959 and Feldman and Reider 1973). In the present paper, an analysis has been made of 18 families carrying various combinations of these two genes and their effects on the percentages of the different haemoglobins.

Materials and methods

Haemoglobin analysis was carried out in the following way on four families (IA to IVA) of β-thalassaemia homozygous and fourteen (IB to 14B) of HbE-β-thalassaemia patients (table 1).

Table 1. Total individuals studied of the β-thalassaemia homozygous and HbE-β-thalassaemia families.

Type	No. of families	No. of family members	No. of sibs
β-Thalasaemia homozygous	4	23	15
HbE-β-Thalassaemia	14	64	39
Total	18	87	54

Anticoagulated blood samples were collected. The haemoglobin percentage and blood grouping were determined and the sickling test carried out (Dacie and Lewis 1968). Haemolysates were prepared by standard techniques for electrophoresis and foetal haemoglobin estimation. Paper, starch and polyacrylamide gels were used as media for comparison. Spectrophotometric quantitations were made after the elution of haemoglobins separated on polyacrylamide gel electrophoresis (Ajmani *et al.*, 1976, 1977).

For the separation of different haemoglobins, a 5% polyacrylamide gel solution was prepared by mixing one part of stock solution A containing acrylamide 20% and methylene bisacrylamide 0.53% and two parts of stock solution B (consisting of tris-HCl buffer—17.15 g tris and 24 ml of N.HCl/dl, pH 8.8 and 0.4 ml N, N, N′, N′ tetramethylene diamine and one part of aqueous ammonium persulphate solution 0.48%. Gel solution was immediately pipetted into glass gel tubes (12×0.5 cm). Bridge buffer pH 8.3 (Tris 0.6% and glycine 2.88%) was poured in both the electrode chambers and polyacrylamide gel electrophoresis was carried out according to Davis (1964) and a current of 4 mA per tube was applied for a period of 120 min at 22°C. Two distinct bands appeared, a major band (HbA) and a minor one (HbA_2 or HbE) which moved slightly behind it; both were distinctly visible without further staining. The bands were cut out and placed in Drabkin's solution. The cyanmethaemoglobin contents of the respective haemoglobins were measured at 420 nm in a Beckmann spectrophotometer (model DU_2). Foetal haemoglobin was measured by the alkali denaturation technique of Betke *et al.* (1959). HbA_2 was considered negative (−) when not visible and hence not detectable by our method.

Results

Pattern of HbA_2 and HbF in β-thalassaemia homozygous families

The sickling test was negative in all the cases. Table 2 shows the distribution pattern of haemoglobins—HbA_2 and HbF of the members of four β-thalassaemia homozygous families.

Table 2. Distribution pattern of HbA_2 and HbE in β-thalassaemia homozygous families

	β-Thalassaemia homozygous	β-Thalassaemia heterozygous	Normal
No. of individuals	5	18	0
HbA_2%	3.45±1.38	7.31±2.16	0
HbF%	41.77±18.70	1.75±0.16	0

Table 3 shows the distribution pattern of different haemoglobins HbA_2, HbE and HbF among the family members of the 14 HbE-β-thalassaemia families.

Table 3. Distribution pattern of HbA_2, HbE and HbF in HbE-β-thallassaemia families.

	HbE-β Thalassaemia	HbE-β Heterozygous	β-Thalassaemia heterozygous	Normal
No. of individuals	16	17	22	9
HbA_2%	–	–	5.53±1.24	3.17±0.90
HbE %	48.48±10.72	29.51±4.58	–	–
HbF %	31.14±14.26	0.99±0.64	1.66±1.49	1.24±0.90

The age of the patients ranged from 10 months to 17 years and their HbA_2 percentage was always within the normal range. The only exception was family IIA (figure 1) where one of the brothers (818) of the patient was later diagnosed as a β-thalassaemia homozygote with high percentages of both haemoglobins F and A_2. The patients available did not show total absence of adult naemoglobin (HbA). None of the cases received any blood transfusion before the study.

Wherever both parents of the HbE-β-thalassaemia patient were available for studies, one parent was invariably found to be a carrier of HbE-disease and the other of β-thalassaemia. Amongst the brothers and sisters of the patients all types of combinations were observed including HbE carrier (HbAE), β-thalassaemia carrier (HbAT), HbE-β-thalassaemia diseased and also normal individuals. The ages of the patients ranged from 1 year and 10 months to 28 years with total haemoglobin percentages between 1.1 and 6.3. A few normal cases, having HbA_2 and HbF in the normal range, were reported as well, except sample 597 of family IB who showed foetal haemoglobin as 3.3% of the total haemoglobin.

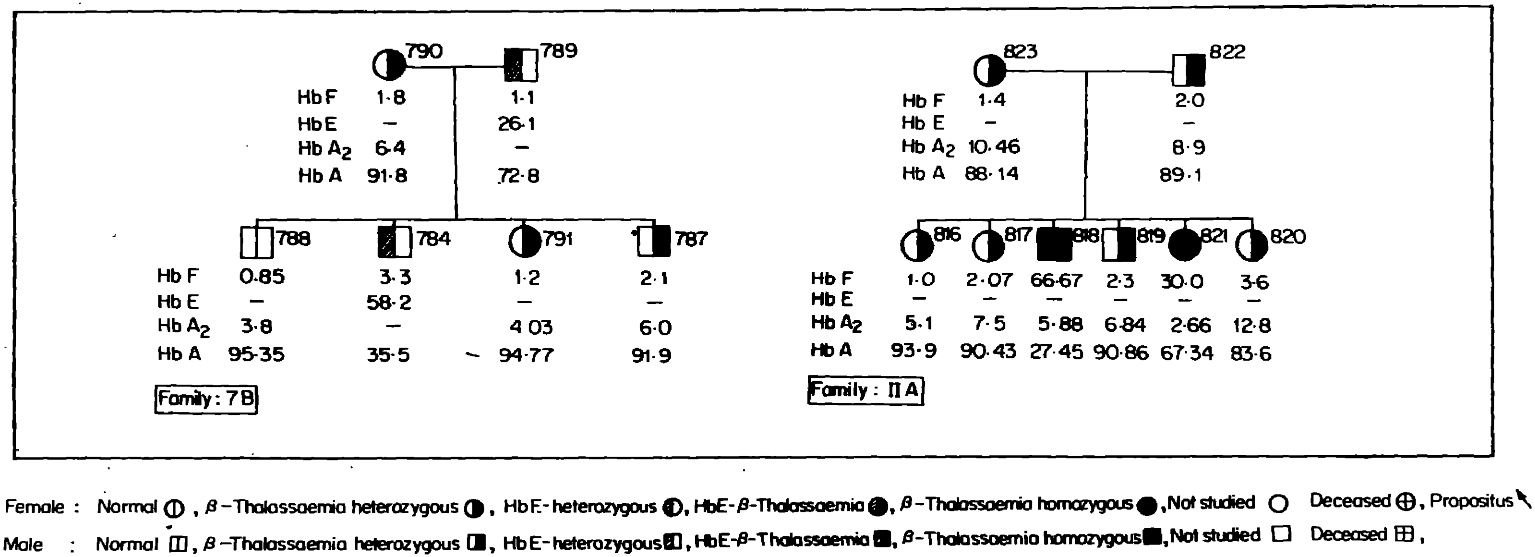
790
789
Hb F 1·8 1·1
Hb E – 26·1
Hb A2 6·4 –
Hb A 91·8 72·8
788
784
791
787
Hb F 0·85 3·3 1·2 2·1
Hb E – 58·2 – –
Hb A2 3·8 – 4 03 6·0
Hb A 95·35 35·5 94·77 91·9
Family : 7B
823
822
Hb F 1·4 2·0
Hb E – –
Hb A2 10·46 8·9
Hb A 88·14 89·1
816
817
818
819
821
820
Hb F 1·0 2·07 66·67 2·3 30·0 3·6
Hb E – – – – – –
Hb A2 5·1 7·5 5·88 6·84 2·66 12·8
Hb A 93·9 90·43 27·45 90·86 67·34 83·6
Family : II A
Female : Normal ◐, β-Thalassaemia heterozygous ◐, HbE-heterozygous ◐, HbE-β-Thalassaemia ●, β-Thalassaemia homozygous ●, Not studied ○ Deceased ⊕, Propositus ↖
Male : Normal ◫, β-Thalassaemia heterozygous ◧, HbE-heterozygous ▨, HbE-β-Thalassaemia ■, β-Thalassaemia homozygous ■, Not studied ☐ Deceased ⊞,

Figure 1. Pedigree analysis and haemoglobin composition of two typical families.

In family 4B, a patient (771) showed total absence of adult haemoglobin. This was the only case available with complete lack of adult haemoglobin. In family 6B, a patient (763) had a very low percentage of haemoglobin A (5.2). Family 7B had the usual pattern in the parents, one of them being a carrier of HbE and the other of β-thalassaemia (figure 1). The patient had a very high percentage of HbE (higher than HbE carrier) with little increase in the percentage of foetal haemoglobin (3.3). A brother (787) and a sister (791) carried β-thalassaemia but another brother (818) had relatively normal haemoglobin components.

Discussion

In the expression of β-thalassaemia two types of genes are involved. The gene β^{o} supresses β^{A} globin formation and results in the complete absence of HbA in homozygotes while the β^{+}gene causes a decrease in the production of β^{A} globin but not its complete suppression (Conconi *et al.*, 1970; Weatherall and Clegg 1972). Earlier Chernoff *et al.* (1956), Wasi *et al.*, (1969) and Feldman and Reider (1973) suggested that β^{o} type is more common in South east Asia since patients with HbE-β-thalassaemia usually show a complete absence of haemoglobin A.

From the results, as shown in table 1, which compares the percentages of different haemoglobins of the thalassaemia patients with those of HbE-β-thalassaemia, the earlier contention, that HbβE and HbβT interact to result in more drastic symptoms than HbE or HbT heterozygotes, is fully borne out. In all cases the HbE and HbF percentages are increased in the double heterozygotes (figure 1). The only exception is family 7B in which the patient, though diagnosed as a HbE-thalassaemic, shows a lower level of haemoglobin F than other similar cases. Apparently, in this patient the interaction between HbβT and HbBE is either not complete or is influenced by some other factors.

In all the cases of homozygous β-thalassaemia studied here haemoglobin A_2 has been observed to be well within the normal range and below the percentage shown by the thalassaemia carrier of the same family. These observations support those of Weatherall and Clegg (1972), though Chatterjea *et al.* (1959) had earlier recorded an increased level of haemoglobin A_2 (8.0%) in thalassaemia homozygotes as well. The present investigation does not support the effort of Chatterjea *et al.* (1959) except in one case (family IIA, sample 818), where relatively high percentages of both haemoglobins F and A_2 were present in a β-thalassaemia homozygote. In the initial patient of the same family (821), however, haemoglobin A_2 was low though the individual had been diagnosed as a β-thalassaemia homozygote.

In Eastern India, on an average, β-thalassaemia is of quite frequent occurrence. The haemoglobin E gene occurs frequently with HbβT and has been suggested to indicate the mongoloid element in the populations of this region (Flatz *et al.* 1972; Das and Flatz, 1975). As far as the present data show, haemoglobin A is present in rather high quantities even in HbE-β-thalassaemia patients (5.2 to 42.5%). The only exception is family 4B, patient (771), where it is totally absent. This patient had an enlarged spleen which had to be later removed. Taking the criterion that the presence of β° type is denoted by the total absence of haemoglobin A, only in this case it appears that the gene involved is of β° type. In all other families studied here, apparently the gene is of β^{+} type since haemoglobin A has been recorded in appreciable quantities. This observation may indicate that these families do not have any Southeast Asian affiliations.

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A probability model for the number of births in an equilibrium birth process

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Abstract. A probability model for the number of complete conceptions (that is, live births) taking into account foetal wastages, occurring in a couple during a specified period of time (T_0, T_0+T) is developed assuming that the data was collected starting a long time after marriage. A method of estimating some of the underlying parameters is given. The model is applied to data obtained in a Varanasi Survey in 1969-70.

Keywords. Equilibrium birth process; estimation of fecundity; sterility; method of moments.

Introduction

The majority of the probability models (Brass, 1958; Pathak, 1966, 1970; Perrin and Sheps, 1964; Singh, 1961, 1964, 1968) were developed using the renewal theory and dealt with a sequence of occurrences of a defined event such as conception or birth. These models assumed that the female was exposed to the risk of conception at the beginning of the observational period. This assumption is not always true, especially when the data on the number of births per female were collected in a retrospective survey. The information in such situations may relate to the number of births observed for any segment of the reproductive life of the female. Obviously for the same reason, models dealing with the number of births during the period (O, T), where O refers to the time of marriage, are not applicable in such situations.

Sheps *et al.* (1969) have pointed out that if the observations, instead of starting at marriage for each woman, begin at a later date and the process has been in effect for a long time, the distribution of births becomes an equilibrium birth process. Recently, Singh and Yadava (1977) obtained a model for the number of conceptions in an equilibrium birth process. Since data regarding the number of live births are readily available and involve no memory bias, it is better to develop a probability model for number of births in order to estimate fecundity and other fertility parameters.

In the present paper, a model describing the variation in the number of births i an equilibrium birth process has been derived. Since a significant proportion o conceptions results in foetal wastage, it has been taken into account. Thoug fertility parameters vary at the individual level, like the female's age and pregnanc history and also between females, they are assumed to be constant here at both th levels due to the complex nature of the model. The suggested model and th underlying assumptions are given below.

The model

Let $X(T_0, T)$ denote the number of complete conceptions (that is, live births) to female during the period (T_0, T_0+T), where T_0 is measured from marriage, that i the observation starts after a time T_0 from marriage. $X(T_0, T)$ can assume values 1, 2, ... The probability distribution of $X(T_0, T)$ is derived under the followin assumptions:

(i) The female has led a married life from marriage to the end of the observ tional period.

(ii) A conception may result in a live birth or in a foetal wastage. If conception results in a live birth it is called complete, failing which it known as incomplete. Let θ and $(1-\theta)$ be the respective proaabilitie $(0<\theta<1)$.

(iii) The waiting time for first conception since marriage follows an exponenti distribution with a probability density function (PDF)

$$f(t) = \lambda e^{-\lambda t}, \quad t>0, \quad \lambda>0 \qquad (1)$$

and the time between the rth and $(r+1)$th conceptions $(r=1, 2, \ldots\ldots)$ follow displaced exponential distribution with PDF

$$f(t) = \lambda e^{-\lambda (t-h_1)}, \quad t>h_1, \quad \lambda>0, \qquad (2)$$

or $$f(t) = \lambda e^{-\lambda (t-h_2)}, \quad t>h_2, \quad \lambda>0, \qquad (3)$$

depending on whether rth conception is incomplete or complete.

The above assumption makes it clear that if there is a conception at a point o time, no other conception is possible either in h_1 or h_2 units of time according as th conception is incomplete or complete. These periods of temporary sterility ar known as 'rest periods' $(h_1<h_2)$.

(iv) Either a female is exposed (except during a pregnancy or post-partun amenorrhoea period) to the risk of conception throughout the observationa period or she is not exposed to this risk at any time during the interval $(T$ $T_0+T)$. Let α and $(1-\alpha)$ be the respective probabilities.

(v) T_0 is at a considerable distance from marriage.

Under the above assumptions the distribution of $X(T_0, T)$ is given by

$$P_0 = 1 - a + a\left[H_0(T) - H_1(T)\right] \tag{4}$$

$$P_r = a\left[H_r(T) - H_{r+1}(T)\right], \quad r = 1, 2, \ldots\ldots, n-1 \tag{5}$$

$$P_n = a H_n(T), \tag{6}$$

where $H_0(T) = 1$ and

$$\begin{aligned}
H_{r+1}(T) &= \sum_{\text{over } j} \binom{r+j}{j} \frac{(1-\theta)^j \theta^{r+1}}{1+\lambda\bar{h}} \Big[G_1(T,\lambda) - (1-\theta) \\
&\qquad G_2(T,\lambda,h_1) - \theta\, G_2(T,\lambda,h_2)\Big] \\
&\qquad \text{for } T > jh_1 + rh_2 + h_2 \\
&= \sum_{\text{over } j} \binom{r+j}{j} \frac{(1-\theta)^j \theta^{r+1}}{1+\lambda\bar{h}} \left[G_1(T,\lambda) - (1-\theta) G_2(T,\lambda,h_1)\right] \\
&\qquad \text{for } jh_1 + rh_2 + h_1 < T \le jh_1 + rh_2 + h_2 \\
&= \sum_{\text{over } j} \binom{r+j}{j} \frac{(1-\theta)^j \theta^{r+1}}{1+\lambda\bar{h}} G_1(T,\lambda)
\end{aligned}$$

$$\text{for } jh_1 + rh_2 < T \le jh_1 + rh_2 + h_1, \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$
$$j = 0, 1, 2, \ldots\ldots; \quad r = 0, 1, 2, \ldots\ldots, n-1,$$

where

$$\begin{aligned}
G_1(T,\lambda) &= \lambda(T - jh_1 - rh_2) - (j+r) + e^{-\lambda(T-jh_1-rh_2)} \\
&\quad \sum_{s=0}^{j+r-1} \sum_{k=0}^{s} \frac{\left[\lambda(T - jh_1 - rh_2)\right]^k}{\underline{\lfloor k}}
\end{aligned}$$

and

$$\begin{aligned}
G_2(T,\lambda,h_i) &= \lambda(T - jh_1 - rh_2 - h_i) - (j+r+1) \\
&\quad + e^{-\lambda(T-jh_1-rh_2-h_i)} \\
&\quad \sum_{s=0}^{j+r} \sum_{k=0}^{s} \frac{\left[\lambda(T - jh_1 - rh_2 - h_i)\right]^k}{\underline{\lfloor k}}, \quad i = 1, 2.
\end{aligned}$$

The derivation of the probability distribution for $X(T_0, T)$ is given below in a separate section.

Estimation

The model consists of five parameters, h_1, h_2, θ, a and λ. Although with suitable statistical techniques all the model parameters can be estimated simultaneously, it is not advisable using some observed distribution containing only a few cells with significant number of observations. Since the values of some of the fertility parameters are more or less constant from sample to sample, their values can be assumed beforehand on an empirical basis. The procedure of estimation of other parameters can then be developed. In this context, assuming the values of outcome-dependent rest periods h_1 and h_2 and the probability of an incomplete conception θ, a procedure for estimating rest of the parameters λ and α is given below:

If $X_1, X_2 \ldots, X_N$ is a random sample of size N from a population, then

$$E(X) = \sum_{r=0}^{n} r P_r = \alpha U(\lambda) , \tag{8}$$

where $U(\lambda)$ denotes a function of λ. Since the samples are taken from the same population, we have

$$E(\bar{x}) = \alpha U(\lambda) , \tag{9}$$

where $\bar{x}$ is the sample mean. Following Cox and Miller (1967) and Singh and Yadava (1977) an approximate expression of $U(\lambda)$ can be written as

$$U(\lambda) = \frac{\theta \lambda T}{1 + \lambda \bar{h}} \ ; \ \bar{h} = (1-\theta)h_1 + \theta h_2 , \tag{10}$$

so that equation (9) can be written as

$$\bar{x} = \frac{\alpha \theta \lambda T}{1 + \lambda \bar{h}} . \tag{11}$$

Further, let $N_r (r = 0, 1, \ldots, n)$ denote the number of couples who have r complete conceptions in the sample. Equating the observed proportion of couples with $r = 0$ to expression (4) we get

$$\alpha = \frac{N - N_0}{N H_1(T)} \tag{12}$$

Using equations (11) and (12), α and λ, the estimates of α and λ respectively, can be obtained.

Derivation of the model

Under the assumptions stated previously, the distribution of $X(T_0, T)$ becomes independent of the starting point T_0 (Cox and Miller, 1967); henceforth we can write $X(T)$ for $X(T_0, T)$.

Let the conceptions be counted from the point T_0 and let the successive conceptions occur at times $T_0 + T_1$, $T_0 + T_1 + T_2, \ldots$, so that T_1 be the time of first conception from T_0 and $T_r (r>1)$ be the time between $(r-1)$th and rth conceptions. Since T_0 is at a considerable distance from marriage, the above birth process becomes an equilibrium birth process. Hence, the waiting times $T_2, T_3, \ldots,$ are independently and identically distributed but T_1 has a different distribution given by

$$f_1(t) = \frac{1-F(t)}{\mu}, \tag{13}$$

where $F(t)$ and μ are respectively, the distribution function and mean corresponding to the PDF of T_r $(r>1)$ (Cox and Miller, 1967). From assumptions (ii) and (iii), it is easy to verify that

$$\mu = \frac{1+\lambda\bar{h}}{\lambda} \; ; \; \bar{h} = (1-\theta)h_1 + \theta h_2 \tag{14}$$

and the P.D.F. of T_1 is T_1 is

$$f_1(t) = \begin{cases} 1/\mu \quad , & 0<t<h_1 \\ \dfrac{1-(1-\theta)\left[1-e^{-\lambda(t-h_1)}\right]}{\mu} & , h_1<t<h_2 \\ \dfrac{1-(1-\theta)\left[1-e^{-\lambda(t-h_1)}\right]-\theta\left[1-e^{-\lambda(t-h_2)}\right]}{\mu} & t>h_2 \end{cases} \tag{15}$$

Let $F_{j+1}(t/j)$ be the distribution function of X_0, which is the waiting time of $(j+1)$th conception on the assumption that preceding j conceptions are incomplete. Then, clearly

$$X_0 = \sum_{r=1}^{j+1} Tr \tag{16}$$

where T_1 has PDF (13) and $T_2, T_3, \ldots, T_{j+1}$ each has PDF

$$f(t) = \lambda e^{-\lambda(t-h_1)}, \; t>h_1, \; \lambda>0 \; . \tag{17}$$

Since $T_1, T_2, \ldots, T_{j+1}$ are mutually independent random variables, the distribution function $F_{j+1}(t/j)$ is given by

$$F_{j+1} \; (t/j) = \int_0^t F(t-u)\, dF^j \,(u) \tag{18}$$

where $F(t-us)$ is the distribution function of T_1 and $F^j(u)$ is the j-fold convolution of the distribution function associated with the PDF (17). It can be seen that

$$F_{j+1} \; (t/j) = \frac{A_1(t,\lambda)}{1+\lambda\bar{h}} \text{ for } jh_1<t \le jh_1+h_1$$

$$= \frac{1}{1+\lambda\bar{h}}\left[A_1(t,\lambda)-(1-\theta)A_2(t,\lambda,h_1)\right]$$

$$\text{for } jh_1+h_1<t \le jh_1+h_2$$

$$= \frac{1}{1+\lambda\bar{h}}\left[A_1(t,\lambda)-(1-\theta)A_2(t,\lambda,h_1)-\theta A_2(t,\lambda,h_2)\right]$$

$$\text{for } t>jh_1+h_2 \, , \tag{19}$$

where

$$A_1(t,\lambda) = \lambda(t-jh_1) - j + e^{-\lambda(t-jh_1)} \sum_{s=0}^{j-1} \sum_{k=0}^{s} \frac{[\lambda(t-jh_1)]^k}{\lfloor\underline{k}} \tag{20}$$

and

$$A_2(t,\lambda,h_i) = \lambda(t-jh_1-h_i) - (j+1) + e^{-\lambda(t-jh_1-h_i)} \sum_{s=0}^{j} \sum_{k=0}^{s} \frac{[\lambda(t-jh_1-h_i)]^k}{\lfloor\underline{k}}, \quad i=1,2. \tag{21}$$

Since $\theta(1-\theta)^j$ is the probability that there are j incomplete conceptions preceding a complete conception, the distribution function of the waiting time of the first complete conception from T_0 is

$$P[X_0 \leq T] = H_1(T) = \sum_{\text{over } j} \theta(1-\theta)^j \; F_{j+1}(t/j); \qquad j = 0,1,2,\cdots\cdots,[T/h_1] \tag{22}$$

If $\varphi_1(s)$ be the Laplace transform of $H_1(T)$, then

$$\phi_1(s) = \left[\frac{\theta}{\mu s} - \theta(1-\theta)\frac{e^{-sh_1}}{\mu s}\frac{\lambda}{\lambda+s} - \theta^2 \frac{e^{-sh_2}}{\mu s}\frac{\lambda}{\lambda+s}\right] \left[1-(1-\theta)e^{-sh_1}\frac{\lambda}{\lambda+s}\right]^{-1} \tag{23}$$

Now let X_i be the time between ith and $(i+1)$th complete conception ($i>0$). It is clear that the distribution function of X_i ($i>0$) will differ from X_0 only due to the distribution of T_1.

Here

$$X_i = \sum_{r=i(j+1)+1}^{(j+1)(i+1)} T_r, \; (i>0) \tag{24}$$

where $T_{ij} + (i+1)$'s ($i = 1,2,3\ldots$) have the displaced exponential distribution given by (3). Hence the Laplace transform $\varphi_2(s)$, corresponding to the PDF OF X_i ($i = 1,2,\ldots$) is the same as given by Singh *et al.* (1973),

$$\phi_2(s) = \left(\frac{\lambda}{\lambda+s}\right) e^{-sh_2} \left[1-(1-\theta)\frac{\lambda}{\lambda+s} e^{-sh_1}\right]^{-1} \tag{25}$$

Since $X_0, X_1, \ldots$ are independent random variables, the Laplace transform of the waiting time of $(r+1)$th complete conception from T_0 is

$$\begin{aligned}
\phi_{r+1}(s) &= \phi_1(s)\left[\phi_2(s)\right]^r \\
&= \sum_{j=0}^{\infty}\binom{r+j}{j}(1-\theta)\,\theta^{r+1}\left(\frac{\lambda}{\lambda+s}\right)^{j+r}\frac{e^{-s(jh_1+rh_2)}}{\mu s} \\
&\quad -\sum_{j=0}^{\infty}\binom{r+j}{j}(1-\theta)^{j+1}\theta^{r+1}\left(\frac{\lambda}{\lambda+s}\right)^{j+r+1}\frac{e^{-s(jh_1+rh_2+h_1)}}{\mu s} \\
&\quad -\sum_{j=0}^{\infty}\binom{r+j}{j}(1-\theta)^{j}\theta^{r+2}\left(\frac{\lambda}{\lambda+s}\right)^{j+r+1}\frac{e^{-s(jh_1+rh_2+h_2)}}{\mu s}
\end{aligned} \qquad (26)$$

Now if $H_r + 1\,(T)$ be the inverse of (26), we have expression (7) and hence under the given assumptions the model (4), (5) and (6) follow.

Application and results

To test the validity of the suggested model, it has been applied to the data on number of births recorded in a Varanasi Survey of 1969-70. The survey was conducted by the Demographic Research Centre, Banaras Hindu University in about 2200 households scattered in 52 villages of Varanasi (Rural). The reference date of the survey was October 1969. The details of the survey methodology are given in Singh *et al.* (1970).

Table 1 presents the observed distribution of the number of live births in a fixed period of seven years before the reference date to women whose present age was in the range 25-30 years. As the average age at marriage for women in the surveyed area is nearly 15 years and the data relates to women aged 25-30 years, the start of the observational period may be assumed to be at a considerable distance from marriage.

Table 1. Distribution of the observed and expected number of births in the last seven years to women aged 25-30 years in the Varanasi survey.

Number of births	Observed number of women	Expected number of women
0	22	22.0
1	58	63.7
2	174	171.7
3	142	132.0
4	32	35.4
5	3	6.2
Total	431	431.0

$\chi^2 = 3.3$ (degrees of freedom = 3)

Before applying the model to the data, it is necessary to point out the following. An interesting feature of the distribution ot post-partum amennorrhoea (p.p.a) associated with a live birth, in the survey, is that it has two peaks. Thus, if it is assumed that the rest period associated with a live birth h_2 takes two values h_2' and h_2'' and π and (1 π) are the proportion of women with the value of h_2 is as h_2' and h_2'' respectively, the model given by (4), (5), (6) and expressions for estimating λ and α, (11) and (12) respectively are slightly modified as

$$P_r^* = \pi P_r(h_2 = h_2') + (1-\pi) P_r(h_2 = h_2'') \qquad r = 0, 1, 2, \ldots\ldots, n \tag{27}$$

$$\bar{X} = \alpha\theta\left[\frac{\pi\lambda T}{1+\lambda\bar{h}_1} + \frac{(1-\pi)\lambda T}{1+\lambda\bar{h}_2}\right] \tag{28}$$

$$\hat{\alpha} = \frac{N-N_0}{N[\pi H_1(T/h_2 = h_2') + (1-\pi)H_1(T/h_2 = h_2'')]} \tag{29}$$

where $h_1 = (1-\theta)h_1 + \theta h_2'$ and $h_2 = (1-\theta)h_1 + \theta h_2''$ in the expression (14). $P_r(h_2 = h_2')$ and $P_r(h_2 = h_2'')$, $r = 0,1,2,\ldots$, n in the expression (27) above are given by equations (4), (5) and (6) respectively by substituting $h_2 = h_2'$ and h_2''. The meaning for H_1 $(T/h_2 = h_2')$ and H_1 $(T/h_2 = h_2'')$ is similar to that in expression (29).

In order to apply the modified model given by (27), we take $T = 7$ years. Singh and Bhaduri (1971) and Singh (1978) have shown that the two peaks of h_2 occur at 3 months and 12 months respectively. Hence $h_2' = 1.00$ year and $h_2'' = 1.75$ years. Further, we assume $h_1 = 0.50$ years, $\theta = 0.90$ and $\pi = 0.40$. These results also agree with those given in Singh and Bhaduri (1971). Using these values, the estimates of α and λ are obtained, after a few iterations, as

$$\alpha = 0.9644, \qquad \lambda = 0.73.$$

Once the estimates of α and λ are obtained, the expected frequencies are easily calculated. The observed and expected frequencies are presented in the second and third columns respectively in table 1. The insignificant value of λ^2-statistic at 5% level of significance suggests that the model under consideration is a better approximation to the situation.

Conclusions

The present model may be utilized to analyse the data on the number of births in different age segments of a woman's reproductive life, as it considers the situation that some of them may not be susceptible for conception at the time when the investigator started to collect the data. The model may repeatedly be used with some further refinement for finding out the fertility performance of a woman as she passes through different age groups.

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A conception dependent probability distribution of couple fertility

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Abstract. A probability distribution for the number of conceptions during a specified period of time is derived assuming that the fecundability as well as the proportion of incomplete conceptions vary between conceptions. A procedure for finding the estimate of some of the parameters has been outlined. The probability model was tested with the date from the Varnasi survey.

Keywords. Fecundability; complete conception; rest period; fertility.

Introduction

Several workers have derived probability models for describing the variations in the number of conceptions during a given time interval (Dandekar, 1955; Brass, 1958; Singh, 1963, 1964, 1968; Singh and Bhattacharya, 1970, 1971; Sheps and Perrin, 1966). In these models, the fertility parameters such as fecundability as well as the proportion of incomplete conceptions (a conception is called complete if it results in a live birth; otherwise, it is called incomplete) are assumed to be constant throughout the observation period. But empirically, it has been observed that these parameters vary from couple to couple and depend on the age and conception or parity for the same couple. Recently, Singh et al. (1974) proposed a model for the number of births during a few years of married life assuming fecundability to vary with parity. Singh and Singh (1978) extended the above model for conceptions introducing two types of pregnancy outcomes, i.e., complete and incomplete conceptions. In all the above models, the proportion of incomplete conceptions is taken as constant throughout the period of observation. But Gulick (1971) reported that the risk of incomplete conceptions varies according to the order of conceptions and this conception linked risk phenomenon is not geographical but 'biological'. In addition, the age of the mother, income, environmental conditions, educational status and other variables contribute to the increase in incomplete conceptions. These special conception-risks are always present and must be taken into account in derivation of the probability distribution because such a model would be more akin to the human fertility phenomenon and provide a better estimate of fecundability.

The purpose of this paper is to present a probability distribution model for t number of conceptions during a specified period of time, on the assumptions t the fecundability as well as the proportion of incomplete conceptions depend conception.

The probability model

Let $X(t)$, $0<t<T$, denote the number of conceptions during the period $(0, t)$ length t. θ_i $(i = 1,2,\ldots)$ is the probability that the ith conception is complete so th $(1-\theta_i)$ is the probability that it is incomplete. The probability distribution $X(T)$ is derived under the following assumptions:

(i) The female has led a married life throughout the period $(0, T)$.

(ii) Probability that the first conception occurs during the small time interval $(t, t+\Delta t)$ is $m_0\,\Delta t + 0(\Delta t)$, where m_0 is the conception rate in t beginning, $m_0>0, t>0$.

(iii) After a conception, there is no possibility of another conception f constant times h_1 and h_2 where h_1 and h_2 are the rest periods (duration pregnancy and post partum amenorrhoea) associated with incomplete ar complete conceptions respectively.

(iv) Given $X(t) = r$, $r = 1,2,3,\ldots$, the conditional probability of a conceptic during the time interval $(t, t+\Delta t)$ is $m_r\Delta t + 0(\Delta t)$, if the rth conception h occurred prior to $(t-h_2)$ in case of complete and has occurred prior $(t-h_1)$ when it is incomplete conception, zero otherwise, where m_r is t conception rate after the rth conception.

(v) The rest periods and the time intervals of fecundable state are mutual independent.

(vi) Either the female is exposed to the risk of conception throughout the inte val $(0, T)$ except during the rest period; or she is not exposed to this risk any time during the interval $(0, T)$. Let a and $(1-a)$ be the respectiv probabilities.

As explained in Singh and Singh (1978), the maximum number of conceptions b a female during the time interval $(0, T)$ cannot exceed n, where $n = [T/h_1]$ $[T/h_1]=T/h_1$; otherwise, it is equal to $[T/h_1]+1$, where $[T/h_1]$ stands for th greatest integer not exceeding T/h_1, $h_1<h_2$.

Under the assumptions 1,2,3,4,5 and 6, the distribution function $H_r+{}_1(T)$ ca be obtained as

$$H_{r+1}(t) = a\prod_{i=1}^{1_r}(1-\theta_i)\left[B(1-e^{-m_s(t-rh_1)} + \sum_{k_1=1}^{1_r}\frac{\theta_{k_1}}{(1-\theta_{k_1})}\left\{B(1-e^{-m_s(t-h_2-\overline{r-1}h_1)}\right\}\right.$$

$$+\left\{\sum_{k_1\neq k_2=1}^{1_r}\left(\prod_{j=k_1}^{k_2}\frac{\theta_j}{(1-\theta_j)}\right)\right\}\left\{B(1-e^{-m_s(t-2h_2-\overline{r-2}h_1)}\right\} + \cdots\cdots + $$

$$\left\{\sum_{k_1\neq k_2\neq\cdots\neq k_{1_{r-1}}=1}^{1_r}\left(\prod_{j=k_1}^{k_{1_{r-1}}}\frac{\theta_j}{(1-\theta_j)}\right)\right\}\left\{B(1-e^{-m_s(t-\overline{r-1}h_2-h_1)})\right\}+$$

$$\left\{\sum_{k_1\neq k_2\neq\cdots\neq k_{1_r}=1}^{1_r}\left(\prod_{j=k_1}^{k_{1_r}}\frac{\theta_j}{(1-\theta_j)}\right)\right\}\left\{B(1-e^{-m_s(t-rh_2)})\right\}\Bigg] \quad \ldots\ldots\ldots (1)$$

$$\text{where } B=\sum_{s=0}^{r}\left(\prod_{\substack{y=0\\ y\neq s}}^{r}m_y\right)\Big/\prod_{\substack{y=0\\ y\neq s}}^{r}(m_y-m_s)$$

The above model is derived considering all θ_i's ($i = 1,2,3,\ldots,r$) and m_y's ($y = 0,1,2,\ldots,r$) to be distinct. However, if $\theta_1 = \theta_1$, $\theta_2 = \theta_3 \ldots = \theta_r = \theta$ and $m_y = m$ for $y = 1,2,3,\ldots,r$, except m_0, the above model reduces to the following form:

$$H_{r+1}(t)=a\left[(1-\theta_1)\cdot\sum_{j=0}^{1_{r-1}}\binom{r-1}{j}\theta^j(1-\theta)^{r-j-1}\cdot W+\theta_1\sum_{j=1}^{1_r}\binom{r-1}{j-1}\theta^{j-1}(1-\theta)^{r-j}\cdot W\right]$$

where

$$W=\left[\left(\frac{m}{m-m_0}\right)^r(1-e^{-m_0(t-jh_2-\overline{r-j}h_1)})-m_0\left\{\sum_{s=0}^{r-1}\frac{m^s}{(m-m_0)^{s+1}}\cdot\left(1-e^{-m(t-jh_2-\overline{r-j}h_1)}\right.\right.\right.\,.$$

$$\left.\left.\left.\sum_{u=0}^{r-s-1}\frac{\{m(t-jh_2-r-jh_1)\}^u}{u!}\right)\right\}\right] \quad \ldots\ldots\ldots (2)$$

If we replace m by dm_0 where d is constant, equation (2) assumes the following form:

$$H_{r+1}(t)=a\left[(1-\theta_1)\sum_{j=0}^{1_{r-1}}\binom{r-1}{j}\theta^j(1-\theta)^{r-j-1}W'+\theta_1\sum_{j=1}^{1_r}\binom{r-1}{j-1}\theta^{j-1}(1-\theta)^{r-j}W'\right].$$

where

$$W'=\left[1-\left(\frac{d}{d-1}\right)^r\cdot e^{-m_0(t-jh_2-\overline{r-j}h_1)}+e^{-dm_0(t-jh_2-\overline{r-j}h_1)}\cdot\sum_{s=0}^{r-1}\frac{d^s}{(d-1)^{s+1}}\cdot\right.$$

$$\left.\sum_{u=0}^{r-s-1}\frac{\{dm_0(t-jh_2-\overline{r-j}h_1)\}^u}{u!}\right]. \quad \ldots\ldots\ldots (3)$$

Hence the probability of r conceptions during the time interval $(0, T)$ is

$$P[X(T)=r]=H_r(T)-H_{r+1}(T),\ r=1,2,3\ldots\ldots(n-1).$$

$$P[X(T)=n]=H_n(T),\ H_1(T)=a(1-e^{-m_0T}),\ H_0(T)=1.\ \ldots\ldots(4)$$

Derivation of the model

Let successive conceptions occur at times $(Z_1, Z_2,\ldots,Z_r, Z_{r+1},\ldots)$ so that $(Z_{r+1} - Z_r)$ is the time interval between the rth and $(r + 1)$th conception. This interval of time is the sum of two types of interval: (a) a time interval, denoted b S_r, during which no additional conception can occur, that is the rest period following the rth conception and (b) time interval T_r, which is the time between resumption of fecundable state after the termination of rth conception and the time o $(r+1)$th conception. If T_0 is the time of first conception from marriage, the $T_0 = Z_1$ and $S_0 = 0$. From the assumptions 1 and 2, T_0 follows an exponentia distribution (see Singh, 1964), with distribution function

$$P\left[T_0 \leq t\right] = 1 - e^{-m_0 T} \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Now ε_r *and* η_r are the two quantities defined by

$$\epsilon_{r+1} = \sum_{i=0}^{r} T_i \, , \; \eta_r = \sum_{j=1}^{r} S_j \, , \; \eta_0 = 0, r>0 \quad \ldots\ldots (6)$$

It is assumed that each of the two sequences $\{T_i, i \geqq 0\}$ and $\{S_j, j \geqq 1\}$ are mutually independent random variables. Hence $Z_{r+1} = \varepsilon_{r+1} + \eta_r$ would be a set of independent random variables. Since, ε_{r+1} is the sum of $(r+1)$ displaced exponentially distributed random variables, the distribution function of ε_{r+1}, denoted by $F_{r+1}(t)$ can easily be obtained as

$$F_{r+1}(t) = P\left[\epsilon_{r+1} \leq t\right] = \sum_{s=0}^{r} \frac{m_0 m_1 \ldots\ldots m_r \int_0^t e^{-m_s \cdot x} \cdot dx}{\prod\limits_{\substack{y=0 \\ y \neq s}}^{r} (m_y - m_s)} \quad \ldots\ldots\ldots\ldots (7)$$

where all m_y's are distinct (see Singh *et al.*, 1974; Singh and Singh, 1978). From assumption 3, S_j follows two-point distribution, given by

$$\left.\begin{aligned} &P\left[S_j = h_2\right] = \theta_j, \; 0 \leq \theta_j \leq 1 \\ &P\left[S_j = h_1\right] = (1-\theta_j) \, , \; j = 1,2,\ldots\ldots\ldots, r \end{aligned}\right\} \ldots\ldots\ldots\ldots\ldots (8)$$

and hence the distribution function of S_j denoted by $G_r(t)$, as obtained by Singh and Singh (1979) is

$$G_r(t) = P\left[\eta_r \leq t\right] = \prod_{i=1}^{1_r} (1-\theta_i) \left[1 + \sum_{k_1=1}^{1_r} \frac{\theta_{k_1}}{(1-\theta_{k_1})} + \sum_{k_1 \neq k_2 = 1}^{1_r} \left\{ \prod_{j=k_1}^{k_2} \frac{\theta_j}{(1-\theta_j)} \right\} + \right.$$

$$\sum_{k_1 \neq k_2 \neq k_3 = 1}^{1_r} \left\{ \prod_{j=k_1}^{k_3} \frac{\theta_j}{(1-\theta_j)} \right\} + \cdots\cdots\cdots\cdots +$$

$$\sum_{k_1 \neq k_2 \cdots \neq k_{1_{r-1}} = 1}^{1_r} \left\{ \prod_{j=k_1}^{k_{1_{r-1}}} \frac{\theta_j}{(1-\theta_j)} \right\} + \sum_{k_1 \neq k_2 \cdots \neq k_{1_r} = 1}^{1_r} \left\{ \prod_{j=k_1}^{k_{1_r}} \frac{\theta_j}{(1-\theta_j)} \right\} \Bigg]$$

$$\text{where } 1_r \quad \min \left[r, \frac{t - rh_1}{h_2 - h_1} \right] \quad \cdots\cdots\cdots (9)$$

Now the distribution function of $Z_r + 1$, denoted by $H_r + 1\ (t)$ is

$$H_{r+1}(t) = \int_0^t F_{r+1}(t-x)\, dG_r(x) \quad \cdots\cdots\cdots\cdots (10)$$

Using equations (7) and (9) in equation (10), on simplification and considering also the assumption (6), the final expression is obtained.

Application of the model

For showing the utility of the probability model, we must have accurate data for the number of conceptions. Due to non-availability of such data for a specified period of time, the model can be applied to the data on the number of births. For example, its application may be seen, where, one to one correspondence between a conception and a live birth is assumed and the children dying within a year and those surviving for more than a year are accounted separately. In this connection, it should also be noted that the rest period following an infant death is considerably shorter than that occurring when the child surviving at least for one year. A similar situation applies to incomplete and complete conceptions. Therefore, for illustration, the present model is applied to the data collected in the Varanasi Survey 1969-70. A detailed account of the survey is given by Singh *et al.* (1970).

For this suvey, an analysis of birth intervals was done according to the life table techniques, which was similar to that of Sheps (1965) and it revealed that the average period of time in the case of first few births, with the exception of first are approximately the same and these intervals for two consecutive births are comparatively smaller than the interval of first birth from marriage for those females who were married between the ages 13 to 16 years. Similar results have been obtained by Jain (1964) and others. Gulick (1971) reported that the risk of death of a first born child is larger than that for the second, third, fourth or fifth. In accordance with this empirical observation, it is desirable to take $\theta_1 = \theta_1$, $\theta_2 = \theta_3$ $=, \ldots, = \theta_r = \theta$ and $m_y = dm_0 (y = 1,2,3, \ldots, r)$ for illustrative purpose.

Again, if the sample of couples is taken from a population which is mixture of t types of populations, *viz.*, (a) the population of females, which have longer du tion of post partum amenorrhoea when the new born survives more than a year a have shorter duration of post partum amenorrhoea when the new born dies withi year and (b) the population of females, who have shorter duration of post partı amenorrhoea and equal length of intervals whether the new born dies within a yє or survives more than a year. Therefore, the probability model may be express as

$$P\left[X(T)=r\right] = \pi\left[P\left\{X(T)=r/h_1,h_2\right\}\right] + (1-\pi)\left[P\left\{X(T)=r/h_1',h_2'\right\}\right] \ldots\ldots\ldots\ldots(1$$

where $h_1' = h_2' = h_1$ and π and $(1-\pi)$ are the proportion of subgroups.

The present model is applied for the number of births during a period of t years. When any distribution is applied to the observed data, reasonable valu for parameters such as h_1, h_2, θ_1, θ and π can be assumed. Hence $h_1 = 1.00$ year months for gestation and 3 months for post partum amenorrhoea), $h_2 = 1.75$ yea (9 months for gestation and 12 months for post partum amenorrhoea), $\pi = 0.$ (Singh and Singh 1978) and $\theta_1 = 0.75$, $\theta = 0.85$ (Gulick, 1971) are assumed. Tl estimates of the remaining parameters a, d and m_0 are calculated by equating tl mean, variance and relative frequencies of females with zero birth of the observє distribution to their theoretical expression: *i.e.*,

$$\bar{x} = \sum_{r=0}^{n} r.P_r = aY_1(m_0,d) \quad (\text{say}) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(12)$$

$$s^2 = \sum_{r=0}^{n} r^2.P_r - \left\{aY_1(m_0,d)\right\}^2 = Y_2(a,m_0,d) \quad (\text{say})\ldots.(13)$$

$$f_0 = 1 - a + ae^{-m_0T} \ldots\ldots\ldots\ (14)$$

where $\bar{x}$, s^2 and f_0 are the mean, variance and proportion of couples having no birt in the observed distribution respectively. Y_1 (m_0, d) and Y_2 (a, m_0, d) are tb function of m_0, d and a, m_0, d respectively.

For large T, approximate expression for the mean of the distribution similar t those of Brass (1958) and Singh and Singh (1978), can be obtained as

$$\bar{x} = a\left[\pi\left\{1 + \frac{dm_0T'}{1+dm_0\bar{h}(1-\bar{h}/2T')}\right\} + (1-\pi)\left\{1 + \frac{dm_0T''}{1+dm_0h_1(1-h_1/2T'')}\right\}\right]$$

$$\text{where } T' = T-\bar{h}-1/m_0,\ T'' = T-h_1-1/m_0,\ \bar{h} = \frac{(\theta_1+\theta)h_2}{2} + \frac{\{(1-\theta_1)+(1-\theta)\}h_1}{2}$$

$$\ldots\ldots\ldots\ldots\ldots\ldots\ (15$$

Now m_0 can be obtained from (14) for an assumed value of a. By using the equatioı (15) and the values of m_0 and a, an estimate of d is obtained. A set of such estimate of parameters a, m_0 and d denoted by $\hat{a}$, $\hat{m}_0$ and $\hat{d}$ which satisfy the equation (13) i taken as a first approximation. Further, the estimated values of these parameters may be refined according to the equations (12), (13) and (14) respectively. The values of estimates are

$$\hat{a} = 0.977,\ \hat{m}_0 = 0.339,\ d = 1.63.$$

On the basis of these values, the expected frequencies of column 3 of table 1 are calculated. For $\theta_i = \theta$ ($i = 1,2,3,\ldots,r$) the model reduces to that given by Singh and Singh (1978). The same model is also applied using the estimated values of

$$\hat{a} = 0.977,\ \hat{m}_0 = 0.399,\ d = 1.58.$$

The expected frequencies in column 4 of table 1 are calculated on the basis of these values. The values of x^2 shows that the proposed model is a better approximation for the situation. However, other functional form in regard to conceptions, may also produce the better approximation.

Table 1. Correlation of the observed and expected number of women with the number of births during 10 years of marriage.

No. of births	Number	Expected number	
		$\theta_i = 0.75$, $\theta = 0.85$[a]	$\theta = 0.85$[b]
0	36	36.0	36.0
1	62	60.0	62.3
2	143	138.9	145.1
3	165	175.5	172.6
4	101	104.6	100.4
5	41	29.2	
6 and over	2	7.8	33.6
Total	550	550.0	550.0
λ^2	—	1.70	3.00
Degrees of freedom		2	2

[a] According to Gulick (1971).

[b] Present model.

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Functional changes in rat liver tRNA following aflatoxin B_1 administration

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Abstract. Administration of aflatoxin B_1 (3 mg/kg body wt) to rats leads to strong inhibition of the acceptor activity of liver tRNA as measured by charging with [^{14}C]-chorella protein hydrolysate. The maximum inhibition occurs 2 h after treatment. At increasing intervals after treatment, the inhibition appears to be gradually relieved, till control values are restored by 72 h. The charging experiment using several [^{14}C]-amino acids separately shows pronounced inhibition of acceptor activity of all tRNA species, although the degree of inhibition varies with individual species. Preliminary results seem to rule out the possibility of hypermethylation of tRNA or damage to the CCA terminus as probable causes. The resultant functional changes may be attributed to a covalent interaction of aflatoxin B_1-metabolite with tRNA.

Keywords. Aflatoxin B_1; tRNA; acceptor activity.

Introduction

Aflatoxin B_1, a toxic metabolite elaborated by the mold *Aspergillus flavus* is also a potent hepatocarcinogen (Wogan, 1973; Goldblatt, 1969). Though the exact mechanism of its carcinogenic action is not understood, it has been shown to be able to interact with cellular macromolecules (Clifford and Rees, 1969; King and Nicholson, 1969). It has been suggested that 8,9-epoxy aflatoxin B_1 is the ultimate carcinogenic metabolite which is generated by the action of liver microsomal enzymes on aflatoxin B_1 (Swenson *et al.*, 1977), and this highly electrophilic product interacts covalently with cellular macromolecules to initiate the carcinogenic process (Swenson *et al.*, 1974; Martin and Garner, 1977). Since transfer RNA (tRNA) molecules are key components involved in the regulation of cellular growth and differentiation (Weinstein, 1970; Kerr, 1975), it is of interest to study how aflatoxin B_1 interacts with them. An earlier report from this laboratory (Aboobaker and Bhattacharya, 1977) describes how this compound can form weak complexes with rat liver tRNA *in vitro*. Evidence of some degree of covalent binding was also obtained when [^{14}C] labelled aflatoxin B_1 was allowed to react with tRNA in presence of liver microsomes (Aboobaker and Bhattacharya, unpublished). Interactions of this nature *in vivo* could lead to conformational changes and might even bring about functional perturbations in tRNA.

The present communication deals with the effect of aflatoxin B_1 treatment or process of enzymatic aminoacylation of liver tRNA. This reaction, catalyse aminoacyl tRNA-synthetases, is the initial step in the process of stringing toge of amino acid moieties into protein chains. The amino acid acceptor activi considered to be the primary biological function of tRNA.

Materials and methods

Aflatoxin B_1 was obtained from Calbiochem, La Jolla, California, USA. [^{14}C]-An acids (sp. act 60-200 mCi/mmol) and [^{14}C]-chlorella protein hydrolysate (sp. ac m Ci/matom C) were from Isotope Division, BARC, Bombay. Young male W rats (100-150 g) were intraperitoneally injected with aflatoxin B_1 (dissolve dimethyl sulphoxide) at a dose of 3 mg/kg body wt. At various intervals a injection, the animals were killed and total tRNA was prepared from the live phenol extraction, 1M NaCl extraction and purification on DEAE-cellu column (Yang and Novelli, 1971). The purified tRNA was stripped endogenously-bound amino acids by incubation with Tris buffer, pH 8.0 for 1 37°C. A concentrate containing all the amino-acyl-tRNA synthetases required this study was prepared by DEAE-cellulose chromatography of the 105,0 supernatant from normal rat liver (Pearson *et al.*, 1973).

The assay of amino acid acceptor activity of tRNA was performed following method of Yang and Novelli (1971). The reaction mixture (100 µl) containe µmol of Tris-HCl (pH 7.5), 1 µmol of $MgCl_2$, 2 µmol of KCl, 0.4 µmol of ATP, A_{260} unit of tRNA 0.1 µCi[^{14}C]-amino acid (or 0.5 µCi[^{14}C]-chlorella pro hydrolysate) and a saturating amount of the synthetase enzyme. The react mixture was incubated at 37°C for 10 min. Aliquots of 50 µl were pipetted Whatman No. 3 MM filter paper discs which were then washed successively cold trichloroacetic acid (containing 1% casamino acids), ethanol and ether. dried paper discs were then counted for radioactivity in a Beckman Liquid Sci lation Spectrometer (Model LS 100). Rats injected with dimethyl sulphoxide w used for preparation of control tRNA samples. In the case of individual am acis, the values were expressed as pmol of amino acids bound per A_{260} unit of tR

Results

As we were interested in estimating the amino acid acceptance capacity of tR these assays were conducted with limiting tRNA concentration in the presenc an excess amount of enzyme. Under the assay conditions, the acceptor acti was found to be linear for several amino acis upto 2.0 A_{260} of tRNA, and by 20 the aminoacylation reaction reached a plateau.

It was observed that aflatoxin B_1 treatment led to considerable inhibition acceptor activity of rat liver total tRNA when a mixture of amino acids deri from [^{14}C]-chlorella protein hydrolysate was used for charging (figure 1). T effect was, however, transient; this was shown by the gradual reversal of inhibit of aminoacylation when tRNA was isolated at increasing times after aflato treatment. The activity returned to normal level by 72 h, reflecting renew

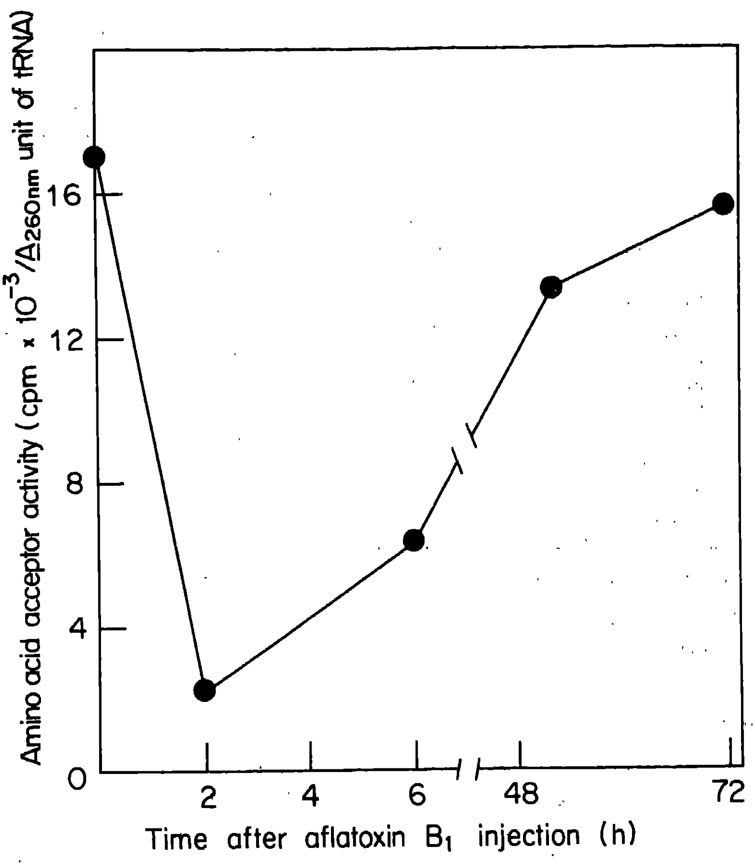

Figure 1. Acceptor activity of liver tRNA from rats at different times after administration of aflatoxin B_1 (3 mg/kg body wt.). Amino acid mixture derived from ^{14}C-chlorella protein hydrolysate was used for charging. Each point represents average value of four rats.

turnover of tRNA species. Further experiments were conducted to determine whether different species of tRNA were affected to similar or different degrees with respect to their individual amino acid acceptor activity. Consequently, liver tRNA was isolated from rats 2 h after aflatoxin B_1 administration, and the individual acceptor activity was measured using 14 different [^{14}C]-amino acids. These results are shown in table 1. In each case, the activity was considerably lower than the corresponding control value. The degree of inhibition varied from 34% to 91%. The most significantly inhibited tRNA species were proline (91% inhibition), aspartic acid (84%) and leucine (80%). Valine, tyrosine, threonine, serine, arginine and alanine-specific tRNA species were inhibited to the extent of about 70%, while tRNAs accepting glutamic acid, glycine, isoleucine, lysine, and phenylalanine were affected marginally (35-40% inhibition).

Table 1. Amino acid acceptor activity of liver tRNA following aflatoxin B_1 administration.

Amino acid	Acceptor activity (pmol/A_{260} unit tRNA)		Ratio
	Control (DMSO)	AFB_1—Treated	Control
Alanine	12.3	4.0	0.32
Arginine	42.0	16.1	0.38
Aspartic acid	24.0	4.0	0.16
Glutamic acid	36.0	16.4	0.45
Glycine	32.0	19.0	0.59
Isoleucine	8.8	4.2	0.48
Leucine	15.0	3.0	0.20
Lysine	12.0	6.4	0.53
Phenylalanine	15.8	10.4	0.66
Proline	27.2	2.4	0.09
Serine	23.0	6.7	0.29
Threonine	12.5	3.3	0.26
Tyrosine	4.5	1.5	0.33
Valine	35.1	10.5	0.30

Rats killed 2 h after administration of AFB_1. Each figure represents average of four animals.

DMSO—Dimethyl sulphoxide
AFB_1—Aflatoxin B_1

Discussion

Among the several biochemical effects produced by aflatoxin B_1, is the stron inhibition it exerts on the biosynthesis of RNA and protein (Wogan, 1973). Sinc aminoacylation of tRNA is among the early steps in the formation of protei chains, interference at this step may well be expected to lead to inhibition o protein synthesis. It is not known at present whether this effect is relevant t carcinogenesis; this might as well be some manifestation of the acute toxic effect o aflatoxin B_1.

It is not clear what mechanism is involved in the inhibition of acceptor activity o tRNA. Changes in the post-transcriptional modification of tRNA may alter it functional property. Hypermethylated tRNA, for example, have decreased amin acid acceptor activity (Bagewadikar and Bhattacharya, 1977). Whether aflatoxi B_1 treatment could bring about alteration in *in vivo* methylation of tRNA was tested by measuring the incorporation of label from injected [$^{14}CH_3$]-methionine int tRNA. Results, however, showed that aflatoxin B_1 treatment had no effect on this process (data not shown). Another possibility is that the carcinogen administration might lead to impairment of the terminal CCA group of tRNA which is absolutely essential for its functional integrity. Transfer RNA with damaged CCA terminus would exert an inhibitory effect on the amino-acylation of normal tRNA if added into the reaction mixture (Herrington and Hawtrey, 1970). However, since, in preliminary studies, it was observed that tRNA prepared from livers of aflatoxin B_1-treated rats failed to produce inhibition of aminoacylation of normal tRNA, destruction of the CCA terminus can be ruled out. This is also confirmed by the observation that inhibition of the acceptor activity cannot be reversed when CTP is added to the aminoacylation reaction system (data not shown).

Certain lines of evidence point to the possibility that the critical target for carcinogens is extragenic (Farber, 1973; Pitot and Heidelberger, 1963; Weinstein, 1970). One such target may well be tRNA. In recent years, several carcinogens have been shown to react with these macromolecular components (Blobstein *et al.*, 1975; Pietropaolo and Weinstein, 1975; Davoud and Griffin, 1976). The findings reported here also seem to be significant from this standpoint. Functional changes have been detected in tRNA molecules after their covalent reaction with certain carcinogenic metabolites (Fink *et al.*, 1970; Fujimura *et al.*, 1972; Bagewadikar and Bhattacharya, 1977). A similar *in vivo* situation may be involved in the case of aflatoxin metabolites. Further work along this line is in progress.

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Chemical modification of bacitracin A and the biological activity of modified bacitracins

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Abstract. The single side chain amino group of the D-ornithine residue in bacitracin seems to be important for the antibacterial activity of the molecule, since, acetylation, formylation, carbamylation and deamination of the antibiotic caused 90-92% loss of antibacterial activity. In contrast, nearly 80-91% of the antibacterial activity of the parent antibiotic was retained after the esterification, amide formation and acid-chloride formation of the α- and γ-carboxyl groups of D-asparagine and D-glutamic acid residues of the antibiotic, respectively.

Keywords. Bacitracin; chemical modification; formylation; acetylation; carbamylation; deamination, esterification.

Introduction

Investigations of the relationships between biological function and structure of proteins and peptides are feasible by chemical modification of reactive groups on these molecules. With peptide antibiotics, such studies can provide a basis for making modifications in which the desirable activities are retained or enhanced and undesirable activities are suppressed or eliminated. Chemical modification studies have been successful in establishing structure-activity correlations for cyclic gramicidins (Stepanov and Silaev, 1961), linear gramicidins (Rambhav and Ramachandran, 1972a, 1972b, 1975, 1976) and polymyxins (Srinivasa and Ramachandran, 1978).

Bacitracins are antibacterial peptides produced by certain strains of *Bacillus licheniformis,* the main component of which is bacitracin A. The antibiotic contains a seven-membered peptide ring with a tail of five amino acids. The bacitracin A molecule has a number of novel compositional and structural features including the thiazoline ring formed between the terminal isoleucine and cysteine residues and an amide bond between the ε-amino group of lysine and asparagine. In addition, like many peptide antibiotics, bacitracin A contains a number of D-amino acids and an ornithine residue. In alkaline solution, bacitracin A is slowly transformed into bacitracin F (Regna, 1959) which has very little antibacterial activity (Hickey, 1964). Hot dilute acid destroys the antibacterial activity, unmasks the thiol group and causes loss of the characteristic absorption band at 253 nm (Wailey, 1966). However, chemical modification of bacitracin has not been studied hitherto, and this paper deals with chemical modification of the amino and carboxyl groups of bacitracin and their effects on the antibact activity of the molecule.

Materials and methods

Bacitracin (58.4 units/mg, Calbiochem, Los Angeles, California, USA) was a sample from Prof. L. K. Ramachandran. All reagents and solvents w commercial samples and purified as and when necessary. Methyl diazoace was prepared by the method described by Searle (1956). Whatmann No. 1 fi paper was employed for paper chromatography.

All colourimetric measurements were done with a Systronics double cell col meter, type No. 102. The content of antibiotic in bacitracin derivatives was ba on weight, and where necessary checked by Pauly's reaction (Pauly, 1915) for imidazole group. Amino groups were estimated by the ninhydrin method (Ro 1957). Acetyl- and formyl group contents in the acetyl and formyl bacitracins w estimated by the method described by Ushalakshmi and Ramachandran (1969a Carbamyl group content in carbamyl bacitracin was determined by the diac monoxime method (Wooten, 1964). The extent of deamination in nitrous a treated bacitracin was monitored by the ninhydrin method. Carboxyl group the antibiotic and its derivatives were estimated by titrating the sample aga standard alkali using 1% alcoholic phenolphthalein as indicator.

Antibacterial activity

Antibacterial activity of bacitracin and its derivatives was determined u *Streptococcus faecalis* (ATCC 8043); and *Staphylococcus aureus* (NCIM 20 two Gram-positive organisms. The growth medium for the test organisms was Henderson-Snell (1948) medium. The pH of the medium was 7.0. Inhibit activity was assayed by adding varying amounts of the antibiotic or derivative in ml of water after sterilizing the tubes containing 5.0 ml of single strength medi Later the tubes were inoculated with the test organisms and the growth of the o nisms after 24 h of incubation at 37°C was measured by turbidimetry in a Systro double cell colorimeter using a red filter. Comparisons of inhibitory activity h been made on the basis of concentrations (μg/ml) necessary to obtain inhibition of growth.

Preparation of modified bacitracins

Formyl bacitracin: To a mixture of 78% formic acid (3 ml) and acetic anhyd (1 ml) in a 15 ml centrifuge tube, bacitracin (60 mg) was added and the pH raised to 4.5 by adding a few drops of 10% sodium hydroxide solution. The t was left in the cold overnight. Later the contents of the tube were diluted to 1 with cold acetone, mixed well and the flocculum, centrifuged. The supernat was discarded and the washing of the residue repeated 2 or 3 times using 15 m cold acetone each time. The final pellet was dried.

Acetyl bacitracin Bacitracin (30 mg) and sodium acetate trihydrate (30 mg) w dissolved in ethanol (2 ml) in a 15 ml centrifuge tube and cooled in an ice ba The reaction mixture was then treated with acetic anhydride (10 μl) at 5 m intervals. The pH of the reaction mixture was maintained at 4.9. A total of 100 µl of acetic anhydride was added and left in the cold overnight. Later, the pH of the reaction mixture was adjusted to 6.5 with dilute sodium hydroxide solution and kept at room temperature for about 15 min. The derivative was precipitated and isolated according to the method given above.

Carbamyl bacitracin Bacitracin (30 mg) and potassium cyanate (17 mg) were dissolved in 2 ml of water in a test tube and the pH was lowered to 6.0 using dilute acetic acid. The reaction mixture was kept in the cold for about 16 h, maintaining the pH at 6.0. The product, carbamyl bacitracin, was precipitated and isolated following the procedure given above.

Deamination of bacitracin An aqueous solution of bacitracin (60 mg in 2 ml) in a test tube was cooled to around 4°C in an ice bath and the pH was adjusted to 5.0 with dilute acetic acid. Gaseous HNO_2 was passed through it for about 20 min after which it was left in the cold for 3 h. Later, the reaction mixture was diluted to about 40 ml with cold acetone to precipitate the product. The product was isolated by filtration.

Bacitracin methyl glycolate An aqueous solution of bacitracin (30 mg in 1.0 ml) in a test tube was cooled to around 4°C in an ice bath and the pH was adjusted to 5.5 using dilute acetic acid. The solution was treated with methyl diazoacetate (10µl) at 5 min intervals. A total of 200 µl of methyl diazoacetate was added maintaining the pH around 5.5 and the temperature around 4°C. The tube was left in the cold overnight. Later, the product was precipitated and isolated according to the method given above.

Bacitracin anilide Bacitracin (30 mg) was dissolved in 1.0 ml of water in a test tube and the pH was lowered to 5.0 using dilute acetic acid and cooled to 0°C. A solution of dicyclohexyl-carbodiimide (DCC) (100 mg in 1.0 ml of water) was added and the mixture stirred in the cold for 30 minutes. Later 1.0 ml of distilled aniline was added and left in the cold overnight. The precipitated product, after adding about 40 ml of cold acetone was isolated by filtration.

Bacitracyl chloride An aqueous solution of bacitracin (30 mg in 1.0 ml) was cooled to about 4° in an ice bath and the pH was adjusted to 5.0 with acetic acid. An aqueous solution of DCC (100 mg in 1 ml) was added and stirred in the cold for about 1 h and then a few drops of 6 N HCl were added to obtain pH 3.0 and the solution left in the cold overnight. The product was precipitated and isolated following the procedure given above.

Results

All bacitracin derivatives, were dried *in vacuo* over solid NaOH. The derivatives were then dissolved in minimal quantity of water, cold acetone added to a slight opalesence and then stored in the cold for several hours to achieve complete precipitation. The precipitated derivatives were filtered off and dried. The yields of different derivatives and the extent of modification in each reaction are shov Table 1.

Table 1. The yields of modified bacitracins and the extent of modification.

Modification	Wt yield of the derivative (mg)	Extent of modification (%)
Acetylation	21.8	97
Formylation	50.2	98
Carbamylation	27.9	98
Deamination	43.0	96
Esterification	25.2	92
Anilide formation	26.2	94
Chloride formation	24.2	92

Chromatographic homogenity of bacitracin derivatives

All modified bacitracins were chromatographed (descending chromatograph n. butanol: acetic acid: water (3:1:1), and gave single spots with Pauly's reag The formyl-acetyl-carbamyl-and deamino bacitracins were ninhydrin-nega The R_f values of acetyl-, formyl-, carbamyl-, and deamino-bacitracins were 0.22, 0.42 and 0.5, respectively. Bacitracin methyl glycolate, bacitracin an and bacitracyl chloride showed R_f values of 0.54, 0.13 and 0.32, respectively. this solvent system bacitracin had an R_f of 0.58.

Antibacterial activity

The results on the antibacterial activity of bacitracin and its derivatives are sh in table 2. The 50% inhibitory concentrations ($I_{50\%}$) for bacitracin in the a using *S. faecalis* and *S. aureus*, were found to be 0.4 µg and 2.0 µg per respectively. Formylation, acetylation, carbamylation and deamination o antibiotic resulted in 90-92% loss of antibacterial activity. Carboxyl gr modified bacitracins, in contrast, were 80-91% as active as bacitracin.

Discussion

Bacitracin is powerfully bactericidal for many Gram-positive bacteria b relatively ineffective against Gram-negative bacteria (Jawetz, 1970). The ins tivity of gram-negative bacteria to bacitracin may be due to the inability o peptide to penetrate the outer membrane. The antibiotic activity of bacitr requires the presence of a divalent cation (Adler and Snoke, 1962).

Table 2. Antibacterial activity of bacitracin and its derivatives.

Compound	*S. faecalis*		*S. aureus*	
	I_{50} % (μg/ml)	Activity[a] %	I_{50} % (μg/ml)	Activity[b] %
Bacitracin	0.4	100	2.0	100
Acetyl bacitracin	4.7	8.5	20.0	10.0
Formyl bacitracin	5.3	7.5	22.0	9.0
Carbamyl bacitracin	4.1	9.7	25.0	8.0
Deamino bacitracin	3.8	10.5	18.0	11.0
Bacitracin methyl glycolate	0.45	88.0	2.2	91.0
Bacitracin anilide	0.45	88.0	2.4	83.3
Bacitracyl chloride	0.48	84.0	2.5	80.0

$I_{50\%}$ Values expressed are the mean values of three experiments.

[a] 0.4 μg/ml is the $I_{50\%} \equiv 100\%$ activity.

[b] 2.0 μg/ml is the $I_{50\%} \equiv 100\%$ activity.

Bacitracin has been reported to affect a number of biochemical processes in bacteria, including the synthesis of inducible enzymes (Smith and Weinberg, 1962); peptidoglycan biosynthesis (Siewert and Strominger, 1962); selective membrane permeability (Snoke and Cornell, 1965); and metal ion transport (Haavik, 1976). Antifungal activity of the antibiotic has also been reported (Venkateswerlu, 1981).

Having known the chemistry, antibacterial activity and the probable mechanism of action of the antibiotic, we have aimed at elucidating the structure-activity correlations for bacitracin. Bacitracin is stable only in the pH range 4.0-6.0. Hence, all chemical modifications performed on the molecule have been carried out between pH 4 and 6, to ensure that stability of the rest of the molecule is unaltered. The property of insolubility of bacitracin in acetone was used to precipitate the derivatives from the reaction mixture. The purity of the derivatives was ascertained by paper chromatography. The degree of modification has been provided based on direct and/or indirect methods. Seven derivatives in all have been prepared. A study of their antibacterial activity has emphasized the essentiality of the amino group, since its removal or blocking results in 90-92% loss of activity. On the other hand, the carboxyl groups appear to be unimportant, at least as regards antibacterial activity, since bacitracin and its carboxyl group modified derivatives are all nearly equally active.

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Combined stress of low environmental pressure and temperature on sodium and potassium homeostasis in rat blood

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Abstract. Adult rats were subjected to the combined stress of low atmospheric pressure and temperature at a simulated altitude of 7620 metres and at 5°C for 5 hours. A significant increase in the influx of rubidium-86 and the efflux of sodium-22 was observed in erythrocytes of experimental rats *in vitro*. Blood 2,3-diphosphoglycerate was appreciably enhanced while blood sodium and reduced glutathione contents in the experimental animals remained unchanged.

Keywords. Hypoxia; temperature stress; ion flux; influx and efflux of ^{22}Na and ^{86}Rb; low temperature; 2,3-diphosphoglycerate; reduced glutathione.

Introduction

In the recent past, comprehensive studies on biochemical aspects of the effects of acute hypoxia (Chandrasekharan *et al.*, 1975; Chander *et al.*, 1975; Namboodiri and Ramasarma 1975, 1978; Ramasarma, 1979; Siddiqui *et al.*, 1979) and cold (Namboodiri and Ramasarma, 1973; Behreus and Hagon, 1974; Kuroshima, 1975) in humans and animals have been carried out. We have earlier reported on biochemical changes induced by acute hypoxia (Sarkar *et al.*, 1977a, b, 1979, 1980a, b) and cold (Sarkar *et al.*, 1981) in the tissues of experimental rats. Since hypoxia lowers the body temperature (Bharat *et al.*, 1979) of laboratory rats and animals exposed to high terrestrial elevation are subjected to the combined environmental stress of low barometric pressures and temperature simultaneously, it is of interest to conduct biochemical studies in experimental animals in the above environmental conditions, to obtain a better understanding of the mechanism of metabolic adaptation at high altitude, the exact sequence of which has not yet been fully elucidated.

The present paper reports studies on the combined stress of low environmental pressure and temperature on sodium and potassium (using ^{86}Rb as the marker) homeostasis in red blood cells *in vitro* and blood 2,3-diphosphoglycerate (DPG) and reduced glutathione (GSH) contents in experimental rats.

Abbreviations used: DPG, 2,3 diphosphoglycerate; GSH, reduced glutathione;

Materials and methods

Adult male Sprague-Dawley rats were exposed to the simulated altitude of 7620 metres at 5°C by keeping them in a standard decompression chamber, with an air flow in the chamber at 2 litres/min. Blood haemoglobin was determined by the standard procedure of Hainline (1958). Influx and efflux of sodium and potassium in erythrocytes *in vitro* were studied using ^{22}Na and ^{86}Rb, according to the method of Dunn and Arditti (1969). Sodium of plasma and red cells was assayed colourimetrically by the method of Weinbach (1935). Blood DPG was measured colourimetrically as per the method of Rose and Liebowitz (1970) and blood GSH was estimated as per the method of Beutler *et al.*, (1963).

Results and discussion

The results in table 1 reveal a significant increase in the influx of ^{86}Rb into red blood cells of experimental rats *in vitro*. A similar increase was also noted in acute hypoxic (Sarkar *et al.*, 1977b) rats, with unchanged values during cold exposure (Sarkar *et al.*, 1981). A significant increase in the efflux of ^{86}Rb observed in the present study denotes that due to combined stress of hypoxia and cold there occurs a loss of potassium from red blood cells. Increase in plasma potassium has also been reported in acute hypoxia (Sarkar *et al.*, 1977b). The loss of intracellular potassium due to diminished oxygen supply (Calkins *et al.*, 1954; Case *et al.*, 1969) has been documented. It is presumed that due to the combined stress of hypoxia and cold, potassium drains out of the red cell. A high concentration of potassium is required to be maintained for the normal metabolism of the red cells. Perhaps this explains the observed increase in the influx of ^{86}Rb.

Table 1. Influx and efflux of rubidium-86 and sodium-22 in erythrocytes of hypoxia and cold-exposed rats.

Group	Influx of ^{86}Rb (cpm/ ml cell)	Influx of ^{22}Na (cpm/ ml cell)	Efflux of ^{86}Rb (cpm/ ml cell)	Efflux of ^{22}Na (cpm/ ml cell)
Control at 30°C	31028±803	1752±90	6225± 95	4672±175
Experimental at 5°C for 5 h at 7620 metres	39535±753^{b}	1625±58	8560±110^{b}	4080±129^{a}

Data represent mean ±SEM of 6-8 animals

^{a} $P<0.02$, ^{b} $P<0.001$

In the present series of experiments, no change was observed in the influx of ^{22}Na into the red cells and this is similar to the observations in conditions of hypoxia (Sarkar *et al.*, 1977b) and cold exposure (Sarkar *et al.*, 1981). But the present study shows decreased efflux of ^{22}Na in red cells *in vitro*, in contrast to the situation of hypoxia (Sarkar *et al.*, 1977b) and cold (Sarkar *et al.*, 1981), where it remained unaltered. The significance of the above findings is not understood at present. The present study does not reveal any change in plasma and red cell sodium, similar to the findings on cold exposure (Sarkar *et al.*, 1981). In contrast, blood potassium homeostasis was considerably disturbed due to the combined stress of acute hypoxia and cold. Hence the response of blood potassium homeostasis to the combined stress of low environmental pressure and temperature is similar to acute hypoxic exposure (Sarkar *et al.*, 1977b).

No change in haemoglobin was observed in the present investigation. The physiological function of DPG in mammals is to reduce the oxygen affinity of haemoglobin and thus help to deliver more oxygen to the tissues (Benesh and Benesh, 1967; Chanutin and Curnish, 1967). Since the elucidation of the above role of DPG in the control of tissue oxygen supply, comprehensive studies indicating significant enhancement of blood DPG in humans and animals in hypoxic conditions have been reported (Lenfent *et al.*, 1968; Torrance and Bartlet, 1970; Rorth *et al.*, 1973, Sarkar *et al.*, 1980b). It is also known that GSH also shifts the dissociation curve of haemoglobin to the right (Horhesi, 1967), thus helping to deliver more oxygen to the tissues, although the capacity of GSH is much lower than DPG. Since DPG in blood regulates potassium homeostasis (Gordos, 1966) and blood DPG and GSH were significantly increased in acute hypoxia, it will be of importance to study the same in blood, subjected to combined stress of acute hypoxia and cold. Thus results in table 2 reveal that blood DPG was significantly enhanced, as in acute hypoxia exposure (Sarkar *et al.*, 1980b) and low environmental temperature (Sarkar *et al.*, 1981). Hence it is apparent that the enhancement of blood DPG as observed in the present study is an adaptive mechanism due to the combined stress of acute hypoxia and cold.

Table 2. Sodium, DPG and GSH levels in the blood of hypoxic and cold exposed rats.

Group	Haemoglobin (g/100 ml blood)	Sodium (meq/litre)		DPG (μmol/g Hb)	GSH (mg/100 ml blood)
		Plasma	red cells		
Control at 30°C	16.56 ± 0.45	127.4 ± 5.5	25.3 ± 2.2	13.6 ± 0.4	43.13±2.4
Experimental (at 5°C for 5 h at 7620 metres)	17.62 ± 0.46	124.2±2.7	28.1 ± 1.8	21.3 ± 1.1[a]	47.5 ± 2.1

(Data represent mean ±SEM of 6-8 animals)

[a] $P<0.001$

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Biochemical studies on the origin of the ATPase of the avian myeloblastosis virus

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Abstract. The adenosine triphosphatase activity of the avian myeloblastosis virus obtained from the blood of the virus-infected chicken was compared with that of the host cell myeloblasts. The specific activity of the viral enzyme is unusually higher than that of the myeloblasts. A significant difference in inhibitor sensitivity was observed with quercetin. When the virus was grown in chicken embryonic fibroblasts in culture, the resulting virus showed very little adenosine triphosphatase activity, comparable to that of the fibroblasts and similar sensitivity to inhibitors. Antibody raised against the purified enzyme of avian myeloblastosis virus inhibits the enzyme activity of the myeloblasts while the activity of the fibroblasts enzyme as well as that of fibroblast-grown virus remains unaffected.

Keywords. ATPase; avian myeloblastosis virus.

Introduction

Mommaerts *et al.* (1952) were the first to demonstrate an adenosine triphosphatase (ATPase) activity in the plasma of birds with induced myeloblastic leukaemia. Later on, Beard (1963) observed that the enzyme activity was associated with the virus present in the plasma. De-The' *et al.* (1964b) localized the enzyme by histochemical techniques, on the viral envelope and on the cell membrane of the myeloblasts where the virus matures. They also observed that in kidney tumours induced by the same virus, neither the cell membrane nor the virus showed any ATPase activity (De The' *et al.*, 1963a). The consensus is that the enzyme is not virus-specific but is incorporated into the virus from the host cell, myeloblasts, during cytoplasmic budding (Haddad *et al.*, 1960). Recently, Banerjee and Racker (1977) solubilized and partially purified an ATPase from virus-infected myeloblasts. The enzyme shows similarity to the cell-surface ATPase of the myeloblasts with respect to its molecular, catalytic and immunological properties (Banerjee, 1978a, 1979a). In order to know the origin of the enzyme, the virus was allowed to grow both in chicken myeloblasts and in chicken embryonic fibroblasts in culture. The ATPase activities of the host cells and the resulting virus as well as their relative sensitivity to some classical inhibitors were studied. The results of these studies are presented in the present communication.

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Materials and methods

Mersalyl was a product of K and K Laboratories, Plainview, New York, USA, N, N′-dicyclohexyl-carbodiimide (DCC) was obtained from Schwarz Biochemicals, Orangeburg, New York, USA. Sodium azide, ATP and ouabain were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Quercetin was procured from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA. Mitochondrial inhibitor of ATPase from bovine heart mitochondria was prepared as described (Kanner *et al.*, 1976).

Myeloblasts from chicken, infected with the avian myeloblastosis virus (AMV) was kindly provided by Dr J. W. Beard and G. E. Houts authorized by the office of Resources and Logistics of the Virus Oncology Program. After infection, the myeloblasts were collected by centrifugation of the blood of the leukaemic chicken at low speed and washed free of virus, if any. The virus was purified from the plasma as described earlier (Banerjee and Racker, 1977). Chicken embryonic cells were cultured as described by Vogt and Ishizaki (1965) and infected with the AMV and grown according to the procedure of Bissel *et al.* (1972). About 150 ml of the culture medium was centrifuged at 50,000 *g* for 15 min in a 30 Ti rotor in a Beckman ultracentrifuge and the supernatant fraction was further centrifuged at 100,000 *g* for 1 h in the same rotor. The pellet was suspended in 3 ml of 10 mM Tris-HCl buffer, pH 7.2 and centrifuged at 105,000 *g* for 30 min. The virus pellet was resuspended in 10 mM Tris-HCl buffer pH 7.2 and used for enzyme assay. The ATPase of AMV and myeloblasts were solubilized and purified as reported earlier (Banerjee and Racker, 1977; Banerjee, 1979a).

For solubilization of the cell-surface ATPase from chicken embryonic fibroblasts, the cells were harvested from 40 tissue culture dishes and suspended in a suitable volume of 0.25 M sucrose, 0.5 mM EDTA, 1 mM ATP and 10 mM Tris-HCl buffer pH 8.0. The cells were centrifuged down at 5,000 *g* for 10 min in a Sorvall RC-2B centrifuge and resuspended in 5 ml of the above buffer. The cells were then stirred in the presence of 200 ml of ice-cold absolute ethanol for 30 min at 0°C to inactivate the mitochondrial or Na^+, K^+-ATPase activities and finally centrifuged at 50,000 *g* for 20 min in a Sorvall centrifuge. The pellet was suspended in 10 ml of the above buffer except that the pH was adjusted to 10. Aliquots of 5 ml were sonicated in a bath type sonicator (T-80-80-I-RS) for 4 min, centrifuged at 160,000 *g* for 1 h and the supernatant fractionated by adding solid ammonium sulphate. The precipitate appearing at 0-60% saturation, was centrifuged at 40,000 *g* for 10 min and suspended in 4 ml of the above buffer at pH 8.0. This preparation was used as a soluble cell-surface ATPase from the fibroblasts.

ATPase activity was measured by determining the inorganic phosphate liberated from ATP according to the method of Taussky and Shorr (1953) and expressed as µmol of P_i formed per min per mg protein. The incubation mixture contained, in a final volume of 0.5 ml, 50 mM Tris HCl buffer pH 8.0, 5 mM M_gCl_2 or $CaCl_2$, 5 mM ATP and a suitable amount of the enzyme preparation. Incubation was carried out at 37°C for 10 min and the reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid. Phosphate was assayed in the supernatant after centrifugation. Protein was determined according to Lowry *et al.* (1951).

An antiserum was prepared by injecting into rabbits subcutaneously, 0.8 mg of purified AMV ATPase in Freund's adjuvant followed by a booster injection of 0.3 mg after 3 weeks. Blood was collected 1 week after the last injection. On an Ouchterlony gel diffusion plate (Ouchterlony, 1958), the purified enzyme showed one very strong precipitation line and a second thin line as shown earlier (Banerjee and Racker, 1977).

Results

The specific activity of AMV ATPase was about 30 μ mol/min/mg protein (table 1). This is the same specific activity reported for the most active preparation of Na^+, K^+-ATPase of plasma membrane (Jorgensen, 1975), or of the Ca^{2+}-ATPase of sarcoplasmic reticulum (MacLenan, 1970; Banerjee *et al.*, 1979) or of the ATPase isolated from the chloroplast (Lien and Racker, 1971). It is several times greater than myosin ATPase and about one-third the activity of the most active preparation of beef heart mitochondrial (F1) ATPase (Pullman *et al.*, 1960). The ATPase activity of the myeloblast, on the other hand, collected from the same virus-infected chicken was as low as 0.26 or even lower (about 1% of the activity of the viral

Table 1. Comparison of the catalytic acnvities of the ATPases of the avian myeloblastosis virus and host cell myeloblasts.

Addition	ATPase activity (μmol P_i/min/mg)			
	AMV	Myeloblasts	Purified enzyme of AMV	Purified enzyme of myeloblasts
None	33	0.26	59	1.1
Mersalyl, 1 mM	7.5		20	0.53
Azide, 2 mM	32.6	0.11	59	1.1
Mitochondrial inhibitor, 50 μg/ml	33	0.18	58	1.15
DCC, 125 μM	6	—[e]	18	0.66
Ouabain, 1mM	32	—	59	1.1
Trypsin[a]	9	—	24	0.7
ATP+Trypsin	30	—	78	1.2

A suitable amount of the virus or the cells or the purified enzyme was preincubated with the indicated concentration of the inhibitor at 37°C for 5 min in the presence of 0.2 ml of 0.25 M sucrose, 0.5 mM EDTA and 10 mM Tris-HCl pH 8.0 (SET buffer). The reaction was started by adding 0.5 ml of the solution containing 50 mM Tris HCl pH 8.0, 5 mM each of ATP and Ca^{2+}

[a] In the case of trypsin, the ratio of protein to trypsin used was 2:1.

[b] The enzyme was protected from the trypsin effect by adding 20 mM ATP.

[c] Not checked.

enzyme), as the preparation was contaminated with azide-sensitive as well as n chondrial inhibitor-sensitive ATPase as shown in the table (viral ATPase is sensitive to azide or the mitochondrial inhibitor). When the myeloblasts were tre with 98% ethanol, the mitochondrial or Na$^+$, K$^+$-ATPase activity, if any, inactivated leaving only the myeloblastic cell-surface ATPase having spe activity of around 0.1 (data not shown) which is at least three hundred times active than the viral ATPase. When the active ethanol precipitate was solubil by sonication at alkaline pH and partially purified, the resulting enzyme prepara showed the same type of sensitivity to the inhibitors (as shown in table 1) w compared with that of the virus or the purified enzyme of the virus. The o molecular, catalytic and immunological properties were also found to be sam reported earlier (Banerjee, 1979). Thus, the most notable feature of the enzyme is the very high specific activity in comparison to the mycloblast enzym the host. The data suggests that the virus may pick up an ATPase from its host in a highly modified state with an increased catalytic ability as shown by the specific activity, without altering its sensitivity to the inhibitors like mersalyl, DC trypsin.

An interesting observation was made regarding the sensitivity of the viral myeloblastic ATPase to quercetin. Table 2 shows that viral ATPase was inhib by quercetin when ATP hydrolysis was measured in the presence of either Mg^{2} Ca^{2+} The sensitivity of the enzyme to quercetin was considerably increased in presence of Ca^{2+} using Mg-ATP as the substrate. In contrast, neither the my blastic ATPase nor the purified enzyme from the myeloblasts was inactivate quercetin. When the viral enzyme was solubilized and purified, it lost the querc sensitivity and behaved like the myeloblastic ATPase.

Table 2. Effect of quercetin on the ATPase activity.

Enzyme	Addition	ATPase activity (μmol/P$_i$/min/mg)		
		Mg-ATP	Ca-ATP	Ca^{2+}+MgATP
AMV ATPase	None	23	30	24
	Quercetin[a]	14	20	5.5
Myeloblast ATPase	None	—[b]	—	0.26
	Quercetin	—	—	0.24
Purified myeloblast ATPase	None	0.9	1.1	0.9
	Quercetin	0.9	0.95	0.85
Purified AMV ATPase	None	60	80	64
	Quercetin	62	80	55

ATPase activity was assayed in the presence or the absence of quercetin after preincubati 37°C for 5 min as described in the legend to table 1. In the case of column 3, preincubation carried out in presence of 5 mM Ca Cl$_2$. ATPase activity was then assayed either with 5 each of ATP and Ca or MgCl$_2$.

[a] The concentration of quercetin used was 20 μg/ml.

[b] Not checked.

To confirm the finding that the viral ATPase is a host-specific enzyme, the virus was allowed to infect and grow in chicken embryonic fibroblasts in culture. The fibroblast cells when assayed for ATPase activity were found to have very low activity and a part of it was contaminated with azide-sensitive ATPase (table 3).

Table 3. ATPase activity of the chicken embryonic fibroblasts.

Addition	ATPase activity (μmol P_i/min/mg)	
	Fibroblasts	Solubilized enzyme
None	0.048	0.1
Mersalyl, 1 mM	0.008	0.005
Azide, 2 mM	0.03	0.009
Quercetin, 10 μg/ml	0.04	0.009
DCC, 125 μM	0.014	0.006
Ouabain, 1 mM	0.05	—
Trypsin	0.022	—
ATP+trypsin	0.042	—

ATPase activity of the fibroblasts or the solubilized enzyme from it was assayed in the presence or absence of the inhibitors according to the procedure as described in the legend of table 1.

Like myeloblast cell-surface ATPase, the fibroblast surface ATPase was also inhibited in the presence of mersalyl or DCC. Inactivation by trypsin and protection with ATP was also the property seen in the case of the myeloblastic ATPase. Ouabain was, however, ineffective. When solubilized, the specific activity of the preparation dropped considerably, probably, due to inactivation of the azide-sensitive ATPase by ethanol and also due to partial inactivation of the surface enzyme or loss of some activating component, if any. The solubilized enzyme was also inhibited by mersalyl and DCC but not affected by azide or quercetin.

Table 4. ATPase activity of the virus grown in chicken embryonic fibroblasts.

Addition	ATPase activity (μmol/P_i/min/mg)
None	0.06
Mersalyl, 1 mM	0
Azide, 2 mM	0.057
Quercetin, 20 μg/ml	0.052
DCC, 125 μM	0
Ouabain, 1 mM	0.06
Trypsin*	0
ATP+trypsin*	0.10

ATPase activity of the virus grown in chicken embryonic fibroblasts was assayed in the presence or absence of the inhibitor according to the procedure described in the legend of table 1.

When the virus was grown in the fibroblasts, and the ATPase activity of t resulting virus was assayed, it demonstrated very low activity comparable to that the fibroblasts as shown in table 4. Sensitivity of the enzyme to the inhibito remained almost the same as that of the host cells except that the enzyme beca more sensitive to mersalyl, DCC and trypsin. The important difference that coul be noticed is the specific activity of the ATPase when the virus was grown in fibr blasts. Unlike the viral ATPase grown in myeloblasts, the enzyme of the vir grown in fibroblasts was not modulated to show very high specific activity and w not inhibited in the presence of quercetin.

The qualitative similarity of the ATPase activity of AMV as well as of its ho cells as regards the sensitivity towards inhibitors, led us to investigate the imm nological properties of the enzymes. The antiserum raised against the purifie viral ATPase inhibited the ATPase activity not only of the virus or of the purifie viral enzyme but also of the myeloblast and the enzyme purified from it, as shown table 5. However, the ATPase activity of the chicken embryonic fibroblast and the solubilized enzyme obtained from it as well as that of the virus grown in fibr blasts were not inhibited by the antiserum when treated under identical condition Incidentally, it should be mentioned that the antiserum crossreacted with th soluble enzyme from myeloblasts but not with that of the fibroblasts in a Ouchterlony gel diffusion test.

Table 5. Effect of antiserum on the ATPase activity.

	ATPase activity (μmol/P$_i$/min/mg)				
	AMV	Purified enzyme from		Solubilized enzyme from fibroblasts	Virus enzyme grown in fibroblasts
		AMV	Myeloblast		
Normal serum	26	80	1.1	0.015	0.062
Antiserum	13	40	0.55	0.015	0.06

A suitable volume of the virus, or the cell or the purified enzyme was preincubated with 20 μl o normal serum or antiserum at 37°C for 75 min followed by the assay of ATPase activity a described in the legend of table 1.

Discussion

Histochemical evidence shows that the avian myeloblastosis virus ATPase is not virus-specific enzyme but originates from the host cells (Haddad *et al.*, 1960). I leukaemic thymuses, the enzyme is localized at the cell membrane where th virus matures (De The' *et al.*, 1963b). When the virus is grown in chondrocyte devoid of any ATPase, the resulting virus does not show any ATPase activity bu contains cartilage fibrils which are the component of the cell membrane of th chondrocytes (De The' *et al.*, 1962). Thus it appears that the cell membrane i involved in the formation of the viral envelope. Similar host cell enzymes hav also been demonstrated in herpes virus (Epstein and Hott, 1963) and influenza virus (Ada and Lind, 1961). De-The' (1964a) has presented evidence based on electron microscopic studies that the ATPase of the AMV originates from the cell membrane of the myeloblasts.

The studies presented in this paper support the original finding of De-The' *et al.*, on the distribution of the virus and myeloblast phosphatase activity (De-The' *et al.*, 1964b). We have indicated three important aspects of the host-virus relationship in connection with the viral association of the enzyme. Firstly, the virus when grown in chicken fibroblasts having very poor ATPase activity also exhibits the same enzyme activity indicating that the virus enzyme may be of host-origin. Secondly, the ATPase activity can be modulated in the virus depending on the host cells in which the virus is maturating. In other words, the virus shows some specificity in modulating its ATPase to a highly active state as observed in the case of myeloblasts but not in case of fibroblasts. The allotopic sensitivity (Racker, 1967) of the viral ATPase to quercetin which is lost upon solubilization of the enzyme may indicate that some component either present in the virus or contributed by the myeloblasts to the viral envelope during cytoplasmic budding, may be responsible for the induction of a remarkably high ATPAse activity in the virus, a phenomenon not observed in the case of fibroblasts. Thirdly, the cell-surface ATPase activity of the fibroblasts shows almost similar inhibitor sensitivity to that of the myeloblasts. Insensitivity of the enzymes of both myeloblasts and fibroblasts to the inhibitors of the mitochondrial ATPase or the Na^+, K^+-ATPase as well as their extreme sensitivity to a mercurial like mersalyl are properties often observed in the case of the cell surface ATPase known as 'ecto-ATPase' (Banerjee, 1978a, b; 1979a, b; 1981; De Pierre and Karnovsky, 1974).

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In vitro formation of thyroid hormones from 3,5-diiodothyronine by supernatant of submaxillary gland

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Abstract. Cell-free extracts of submaxillary glands from rat and goat iodinate exogenously added 3,5-diiodothyronine to form 3,5,3'-triidothyronine and thyroxine. This capacity was elevated after either thyroidectomy or exposure of rats to cold (3 ±1 °C) for 5 h. However, there was no further increase of iodination of 3,5-diiodothyronine when the thyroidectomized rats were exposed to cold stress. The submaxillary extracts have the ability to incorporate radioactive iodide into 3,5-diiodothyronine, 3,5,3'-triiodothyronine and thyroxine. The presence of 3,5-diiodothyronine was also detected in the soluble supernatant of submaxillary extract.

Keywords. 3, 5-Diiodothyronine; thyroid hormone biosynthesis; submaxillary gland.

Introduction

Iodination of tyrosine and monoiodotyrosine (monoiodo-tyr) to form monoiodo- and diido-tyr are the intermediate steps in thyroxine biosynthesis. These reactions are known to be catalysed by peroxidases of the thyroid and some other extra-thyroidal tissues, like, submaxillary, lacrimal, mammary and other glands. However, it has not yet been possible to demonstrate the enzymatic formation of thyroxine from 3,5-diiodothyronine ($3,5\text{-}T_2$) *in vitro* in extrathyroidal tissues.

Results from our laboratory have established the existence (Hati and Datta, 1967, 1969) and, later, the purification of KI-stimulated peroxidase from goat submaxillary gland (Mahajani *et al.*, 1973). The enzyme present in rat submaxillary gland was increased several fold on thyroidectomy (Chandra *et al.*, 1977). The increase of the enzyme activity upon thyroidectomy was abolished on administration of thyroxine (T_4) and 3,3',5-triiodothyronine ($3,3',5\text{-}T_3$). However, $3,5\text{-}T_2$ which has no thyroid hormonal activity, also abolished the effect of thyroidectomy on the iodinating enzyme of the submaxillary gland. The effect of $3,5\text{-}T_2$ as described above was unexpected and suggests that $3,5\text{-}T_2$ exerted this effect by being iodinated to $3,3',5\text{-}T_3$ and T_4 *in vivo*.

Abbreviations used: Monoiodo-tyr, Monoiodotyrosine; Diiodo-tyr, Diiodotyrosine; $3,5\text{-}T_2$, Diiodothyronine; $3,5,3'\text{-}T_2$ Triiodothyronine;-tyr,Thyroxine; $3,3'\text{-}T_2$ and $3,5',3'\text{-}T_3$ represent reversed diiodothyronine and triiodothyronine respectively.

The present study was undertaken to investigate the formation of 3,3′,5-T: T_4 from 3,5-T_2 by the cell-free extracts of the submaxillary gland. Our s indicates that the soluble supernatants of both goat and rat submaxillary gl catalyze the iodination of both endogenous and exogenous 3,5-T_2 to form 3,3′ and T_4.

Materials and methods

Materials

Fresh goat submaxillary glands were collected in ice from the local slaughter h and stored at −10°C before use. 3,5-T_2, 3,3′,5-T_3 and T_4, protease (type VI frc *Streptomyces griseus*), and bovine serum albumin were the products of S Chemical Co., St. Louis, Missouri, USA. Tertiary-amyl alcohol was proc from Riedel-Dettäen A. G. Seelze-Hannover, Germany. Dowex-50WXB form, 20-50 mesh) and H_2O_2 were obtained from British Drug House, Bon Na^{131} I was supplied by Bhabha Atomic Research Centre, Bombay. C chemicals used were of Reagent grade.

Animals

To study the effect of cold stress, male rats (MRC strain) weighing 80-1 maintained on normal diet, were divided into two groups; the control group kept at room temperature (28 ±2°C) and the experimental group was exposec temperature of 3 ±1°C for 5 h. The submaxillary glands were then removei the preparation of extracts as described below.

Thyroidectomy was performed by the procedure described by Chandra (1977).

Preparation of extracts

The submaxillary glands from goat or rat (normal or thyroidectomized) collected and a 10% homogenate was prepared in isotonic KCl at 0°C in a Pc Elevhjem homogenizer. The extract was centrifuged at 700 *g* in a Sorvall R refrigerated centrifuge for 15 min and fatty material floating on the superna was removed. The supernatant was centrifuged again for 1 h at 105 000 *g* Spinco Model L Preparative ultracentrifuge and the resulting supernatant solı was used as the source of iodinating activities

Assay of iodinating activity

The activity was assayed by measuring the incorporation of ^{131}I into 3,5-T_2 to 3,3′,5-T_3 and T_4. The incubation mixture contained in a total volume of 3 ml mM citrate-phosphate buffer of pH 5.0; 0.2 mM 3,5-T_2; 0.13 mM KI; 20 µCi of (carrier-free); 0.166 mM H_2O_2 and a suitable volume of the extract containing a 100 µg of protein. The reaction was initiated by the addition H_2O_2 incubated min at 37°C and terminated by the addition of 0.2 ml of 10 mM soc thiosulphate. Other details of experimental conditions are given in the legen the respective figures or tables. For quicker measurement of the iodin activity, half of the thiosulphate-treated incubation mixture was deprotein with 0.3 ml of 50% trichloroacetic acid and centrifuged. A portion of deproteinized supernatant was applied on a Dowex 50W×8 (H^+ form) column (0.8×6 cm) and the unreacted free inorganic iodide was removed by washing the column with 40 ml of deionized water. The amount or organically-bound iodine was measured by determining radioactivity adsorbed to the column by holding it at a fixed point in a solid scintillation gamma counter.

To determine the exact amounts of 3,3′5-T_3 and T_4 formed, 100 µl of the thiosulphate-treated reaction mixture from the other half was applied on one corner of a Whatmann 3 MM chromatographic paper without deproteinization and chromatographed in two dimensions with two different solvents. First the chromatogram was developed in a descending way in tertiary amyl alcohol: ammonia (30:70) solvent system for 24 h and then in the second direction in butanol: 7 N ammonia (1:1) for 9 h in an ascending mode. Iodocompounds were detected and measured by autoradiographic technique as mentioned earlier by Hati and Datta (1969).

Demonstration of the presence of iodothyronines in submaxillary extract

The soluble supernant of submaxillary extract was incubated with ^{131}I and H_2O_2 in a final volume of 3 ml (as described in the assay procedure) in the absence of any added 3,5-T_2. After 2 h of incubation at 37°C, the reaction was stopped by the addition of thiosulphate as mentioned before. The incubation mixture was adjusted to pH 7.4 with Na_2HPO_4 and half of the incubation mixture was subjected to proteolytic digestion (the amount of type VI protease from *S. griseus* being one-third that of the enzyme protein) for 5 h at 37°C. The mixture was extracted thrice with equal volumes of n-butanol. The other half (protease untreated) of the incubation mixture was extracted with butanol in a similar way. The collected butanol layers were concentrated and applied on paper for chromatographic separation and detection of the individual iodothyronines as described in the assay procedure.

Protein was estimated by following the method of Lowry *et al.* (1951).

Results

Formation of triiodothyronine and thyroxine

Figure 1 demonstrates the chromatographic separation of 3,3′,5-T_3 and T_4 which were formed from 3,5-T_2 using a goat submaxillary enzyme preparation. Rat, hamster and human submaxillary extracts also catalysed the iodination of 3,5-T_2 (data not presented). Iodide incorporation into 3,5-T_2 increased linearly with time as well as with increasing concentrations of protein and the rate of incorporation of I^- was about 230 n mol/min/mg protein.

pH Optimum

'The pH profile of 3,5-T_2 iodination (using citrate-phosphate buffer) is biphasic in nature with a sharp trough in between the two crests. Figure 2 shows a drop in iodinating activity between the two pH optima (5.23 and 5.7, respectively) with a pH difference of only 0.47 in the citrate-phosphate buffer. A similar biphasic nature was also obtained in other buffers like acetate and citrate (data not shown). However, we cannot afford any explanation for such a pH profile.

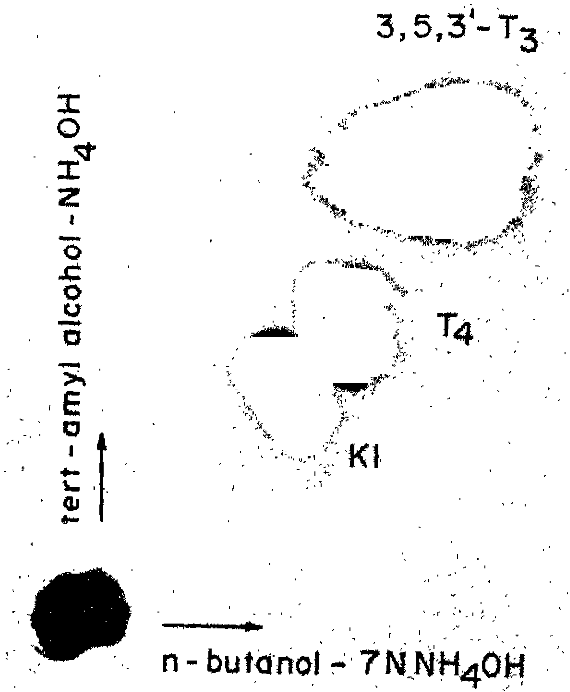

Figure 1. Autoradiogram of the products of the 3,5-T_2 iodination. 3,5-T_2 was iodinated in presence of the reagents, described in Methods under 'Assay of iodinating activity'. After termination of the reaction, 100 µl of the reaction mixture was applied on chromatographic paper for separation and analysis of the products as mentioned in Methods. The spot at the origin indicates protein bound iodine.

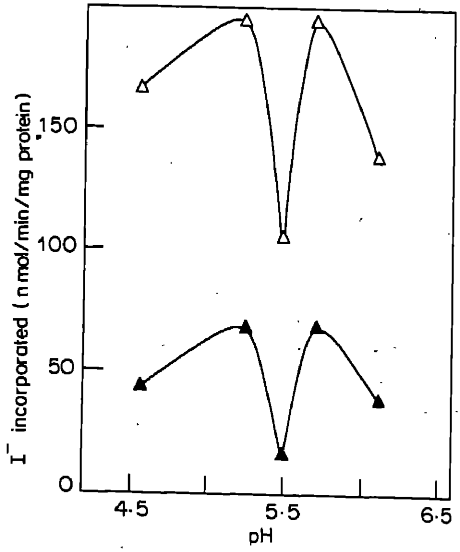

Figure 2. pH optima of the enzyme in citrate-phosphate buffer. In this set of experiments I^- incorporation into 3,3',5-T_3 (△) and T_4 (▲) was measured after paper chromatographic separation of the same as mentioned in Methods.

Effect of varying concentrations of substrates on iodination of 3,5-T_2

The rate of iodination increased with increasing concentrations of 3,5-T_2, KI and H_2O_2 up to about 0.2 mM (data not presented). When one substrate was varied, the other two substrates were kept at their optimum concentration.

In the next experiment, the products of 3,5-T_2 iodination were analyzed after separating them by paper chromatographic technique and the formation of the first product 3,3′,5-T_3 was found to be linear with increasing concentrations of 3,5-T_2 (figure 3).

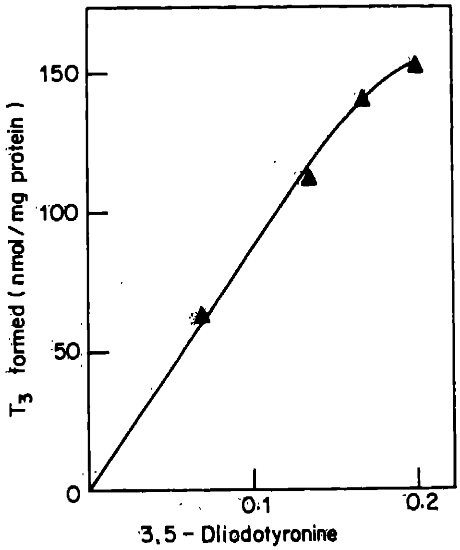

Figure 3. Formation of 3,3′,5-T_3 at varying concentrations 3,5-T_2. Formation of 3,3′,5-T_3 was measured after its separation from other iodo compounds by the paper chromatographic procedure described under Methods. In this experiment, 270 μg of the enzyme extract was used instead of 100 μg.

Effect of various agents on iodination of 3,5-T_2

Table 1 describes the effect of several compounds on rate of iodination of 3,5-T_2; sodium azide was found to be the most potent inhibitor.

Formation of iodothyronines in submaxillary extract

Diiodothyronine iodination observed in the above experiments prompted us to investigate the presence of iodothyronines in the submaxillary extract. Figure 4 demonstrates the incorporation of ^{131}I into iodothyronines, using 3,5-T_2 possibly present endogenously in goat submaxillary extract. The zero time incubation mixture or the incubation mixture containing boiled enzyme preparation treated in a similar way showed only one radioactive spot, that of KI, on the autoradiogram (results not shown). The radioactive spot at the 3,5-T_2 region indicates that the compound was formed possibly by coupling of one tyrosine with another labeled diiodo-tyr. Mono- and diido-tyr did not separate well from KI on the chromatogram under our experimental conditions.

Table 1. Effect of various agents (antithyroid) on diiodothyronine iodination cataly goat submaxillary extract.

Inhibitor	Concentration (μM)	I^- incorporated (nmol/min/mg protein)	Inhibition (%)
None		250.0	
Thiourea	10	117.5	53
	100	0.0	100
Sodium cyanide	100	82.5	67
	1000	40.0	84
Sodium azide	1.0	50.0	80

Goat enzyme (0.22 mg) was used in these experiments. The inhibitors, at the concentr given in the table, were added 10 min before the addition of H_2O_2. The activities measured by the quick assay procedure described in Methods.

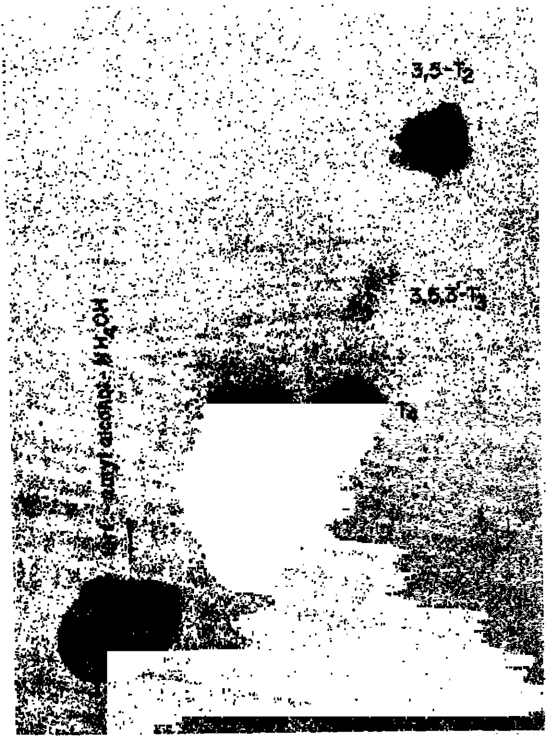

Figure 4. Autoradiogram of the products obtained from endogenous substrates of the maxillary gland.

The figure shows the formation of 3,3',5-T_3 and T_4 from endogenous substrate. demonstrates the presence of iodothyronine in the submaxillary extract (see Methods). autoradiogram was obtained from the incubation mixture, designated as 'protease untreate Methods.

The possibility that 3,5-T_2 is present in the extract of goat submaxillary gland is further strengthened from the data in table 2. It may be concluded from table 2 that most of the 3,5-T_2, 3,3′,5-T_3 and T_4 were bound to a protein in the submaxillary gland which were released upon proteolytic digestion. It could further be suggested that the synthesis of thyroid hormones are facilitated on the protein molecule.

Table 2. Formation of iodothyronines from endogenous substrate(s) of goat submaxillary extract.

System	I^- incorporated (*p* mol/ml submaxillary supernatant)*		
	Diiodothyronine region	Triiodothyronine region	Thyroxine region
Before protease treatment	10.5±0.92	4.5±0.38	3.0±0.84
After protease treatment	44.5±2.22	31.5±3.28	46.0±4.45

Goat submaxillary supernatant 2 ml containing about 9.6 mg protein was used in this experiment.

* Averages of results from three experiments. In these experiments all the reagents except 3,5-T_2, as mentioned in "Assay of Iodinating Activity" were added and the products were analyzed after paper chromatograpic separation.

Detection of 3,5-T_2 in submaxillary gland

To detect the presence of 3,5-T_2 in the submaxillary gland, 30 ml of soluble supernatant containing 249 mg protein was extracted with butanol and chromatographed as indicated before. Although 3,5-diiodothyronine could not be detected in the soluble supernatant, it was detected after the proteolytic digestion of the submaxillary extract. The compound which was sensitive to ferrichloride-ferricyanide-arsenic acid reagent was identical to that of authentic 3,5-T_2 having Rf values of 0.39 and 0.46 in tertiary amyl alcohol: ammonia and butanol: ammonia systems respectively (results not shown).

Effect of thyroidectomy and cold stress on 3,5-T_2 iodination by soluble supernatant of rat submaxillary gland

A previous report from our laboratory already indicated that in rats the monoiodotyr iodination catalysed by the soluble supernatant of the submaxillary gland increased several fold upon thyroidectomy, (Chandra *et al.*, 1977). It was of interest to study the enzyme activity for 3,5-T_2 iodination under identical conditions. The results of table 3 indicate that, although T_3 formation from 3,5-T_2 catalysed by the submaxillary enzyme preparation from a 7 day thyroidectomized animal was not very significant, T_4 formation increased by about 80% follo thyroidectomy. The increase in T_4 formation was observed even 4 days thyroidectomy (data not presented).

Table 3. Effect of thyroidectomy and cold stress on the iodination of diiodothyronine submaxillary extract

System	I^- incorporated (n mol/min/g protein)	
	Triiodothyronine region	Thyroxine region
Normal	119±1.30	40±0.98
Normal+cold Stress	170±0.25	115±0.70
Thyroidectomized	142±2.84	72±0.72

Enzyme protein (200 μg) was used in all the systems. In the second experiment, the ar were kept in cold (3°C) for 5 h, killed and enzyme activity measured in the extr submaxillary gland as mentioned in Methods. The products of 3,5-T_2 in all the above e ments were analyzed after chromatographic separation.

It was reported earlier that there was an increase in peroxidase activity o submaxillary gland upon exposure to cold (Bhattacharyya *et al.*, 1972). Sinc incorporation of iodide into 3,5-T_2 and the peroxidase activity of the submaxi glands both increased upon thyroidectomy, we studied the effect of cold stre 3,5-T_2 iodination by the submaxillary enzyme preparations of normal and th dectomized rats. Table 3 shows that on exposure of a normal rat to cold, the thyroid hormone formation by the submaxillary enzyme preparation was stimul significantly over the control group but that when cold stress was applie thyroidectomized rats, no additional effect was observed (results not shown)

Discussion

We have reported earlier (Hati and Datta, 1967, 1969) that the microsomal frac of the goat submaxillary gland iodinates tyrosine or monoiodo-tyr to proc monoiodo and diiodo-tyr. The evidence presented in this communica indicates that the cell-free extract of goat submaxillary gland, in contrast to thy glands (Taurog, 1964) also iodinates free 3,5-T_2 to produce free 3,3′,5-T_3 an The communication further indicates that the iodinating enzyme present in submaxillary gland is capable of producing thyroid hormone via iodination protein, as proteolysis releases about four-fold more thyroid hormones (tabl Earlier observations of Taurog and Evans (1967) suggested extrathyroidal thyro formation in completely thyroidectomized rats, since they found signifi amounts of thyroxine in the plasma, 2-4 weeks after thyroidectomy. Thu seems likely that extrathyroidal thyroxine formation occurs via the iodinatio both free 3,5-T_2 as well as a protein, although, the site(s) and mechanism of this reaction remain to be elucidated. From different studies it could be suggested that the iodination of tyrosine of monoiodo-tyr in the submaxillary gland has some physiological significance as these reactions are modulated by thyroidectomy (Chandra *et al.*, 1977) or by cold stress (Bhattacharyya *et al.*, 1972). However, none of these studies indicated that thyronine was produced in the submaxillary gland.

The stimulation of 3,5-T_2 iodination upon thyroidectomy confirms the observation of Chandra *et al.* (1977) who showed that monoiodo-tyr iodination catalyzed by a submaxillary preparation increased several fold after thyroidectomy. Thyroid hormone (T_3+T_4) formation from 3,5-T_2 (table 3) also increased after exposure to cold, but there was no further change in T_3 and T_4 formation after exposure of thyroidectomized rats to cold. Increase in the activity under cold stress may depend on the presence of the thyroid gland. These results confirm the observation of Bhattacharya *et al.* (1972).

The scheme suggested by Roche and Michel (1955, 1956) for the synthesis of T_4 from 3,5-T_2 is consistent with our observations with the submaxillary enzyme preparation. However, it has been reported earlier (Alexander, 1961) that thyronine, 3,5-T_2 and 3,3′,5-T_3 are not iodinated by rat or human thyroid extracts. Unpublished results from our laboratory indicate that thyronine is readily iodinated by submaxillary gland extracts and that the product obtained is not identical to 3,5-T_2, 3,3′,5-T_3 or T_4 as its R_f value is different from those of these three iodocompounds in tertiary-amyl alcohol: ammonia (30:70) as well as in a butanol: ethanol: ammonia (5:1:2) system.

Another pathway of T_3 and T_4 synthesis is suggested by the observation that 3,3′-T_2 is formed by coupling of two molecules of monoiodo-tyr catalysed by a beef thyroid extract (Fischer *et al.*, 1964). Formation of labeled 3,3′-T_2 and 3,3′,5′-T_3 (rT_3) *in vivo* in thyroid glands of rat has also been reported by Taurog *et al.* (1976) who suggested that 3,3′-T_2 is formed by coupling of two molecules of monoiodo-tyr and is further iodinated to 3,3′,5′-T_3. The above results imply that 3,5-T_2 is synthesized within the submaxillary gland by coupling of a tyrosine and a diiodo-tyr molecule. According to Roche and Michel (1956) and Kochupillai and Yalow (1978), the formation of 3,5-T_2 is neither due to iodination of thyronine nor to exchange of ^{131}I with the non-labelled iodine molecule of 3,5-T_2. Our results indicate that 3,5-diiodothyronine is a metabolite of the submaxillary gland.

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Effect of nitrogen, phosphorus and potassium deficiency on the uptake and mobilization of ions in Bengal gram *(Cicer arietinum)*

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Abstract. Nitrogen, phosphorus and potassium deficiency reduced the uptake of ^{32}P-phosphate, ^{35}S-sulphate, ^{24}Na-, ^{42}K-, ^{45}Ca-, ^{54}Mn-, ^{59}Fe- and ^{65}Zn- by *Cicer arietinum* (Bengal gram) cv B-75. Root length, leaf area and dry weight of the tissues were also reduced. Since in several cases, the total contents of the radio nucleides both on per plant and per unit dry weight basis were curtailed, the decrease in uptake of several ions cannot be entirely due to reduced growth rate. The reduction in ^{32}P-phosphate uptake was more severe with nitrogen deficient plants than that in phosphate deficient ones; potassium deficient plants, however, took up ^{42}K- as avidly as the control plants. Simultaneously the uptake of $^{35}SO_4^{2-}$ and other cations was affected particularly by nitrogen deficiency. The distribution of radionucleides between the root and shoot portions was also disturbed in several cases by deficiency conditions. The radionucleides taken up accumulated in the young regions as in the case of pea and other dicotyledonous plants. Mobilization of ^{32}P and ^{35}S in the reproductive plants was most markedly affected by nitrogen and potassium-deficiency.

Keywords. Nitrogen; phosphorus; potassium; deficiency; ion-uptake; mobilization; *Cicer arietinum*.

Introduction

The uptake of ions by plants is known to be affected by their deficiencies (Salisbury and Ross, 1974); however, it is not clear how the deficiency of one element affects the uptake of another element. We describe here, some of our observations concerning the effects of three different levels of nitrogen, phosphorus and potassium on the uptake and mobilization of phosphate, sulphate, sodium, potassium, calcium, iron, zinc and manganese ions using radioactively labelled elements in each case. The plant used was Bengal gram (*Cicer arietinum* Linn.), which constitutes an important protein source in the diet of a large section of the Indian population and on the mineral nutrition of which there is very little research reported (Shivraj, 1978).

Materials and methods

Bengal gram (*Cicer arietinum* L.) cv. B-75 was obtained from the Kalyani Seed Farm, Kalyani. The plants were grown in sand culture with three different levels of nitrogen, phosphorus and potassium in porcelain crocks or Brite high density polythene buckets. The normal mineral solution used for growth of plar contained the following in g /l : $NaNO_3$ 7.37; $Na_2HPO_4.12H_2O$ 2.04; K_2SO_4.1. $MgSO_4.7H_2O$ 1.01; $CaCl_2.2H_2O$ 0.30; and traces of micronutrients; pH 6 Solutions were applied to each pot once a week. To obtain deficiency conditic the amounts of nitrogen, phosphorous and potassium in the nutrient solution we reduced to 1/9th of this level.

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Plants were fed with the radioactively-labelled ions through their roots for 3 For anion uptake, young vegetative (4-5 week old) or mature plants (10 week old) fruiting condition were allowed to take up phosphate-^{32}P(5 μCi/ml) or sulphate- (6 μCi/ml) from the nutrient solution. For cation uptake, the following elemei were applied as their chlorides. ^{24}Na (10 μCi/ml), ^{42}K (12 μCi/ml), ^{45}Ca(5 μCi/m ^{54}Mn (5 μCi/ml), ^{59}Fe (7 μCi/ml) and ^{65}Zn (5 μCi/ml). After the experiment, tl roots of the plants were washed first in tap water, then in phosphate buffer co taining the same salt in the non-radioactive form and finally with distilled wat The plants were divided into two groups. The aerial parts of one group were det ched from the roots, and exposed to x-ray films for autoradiography. The root ar shoot tissues of the other group were separated, dried, powdered and radioact vity measured with an end-window counter. For soft β-emitters like ^{35}S and ^{45}C counts were taken in saturation thickness.

Results

Growth

Deficiency conditions affected the growth of the plants as indicated by decrease fresh and dry weights. Although shoot height was only slightly affected, roc length, branch number and leaf area were markedly reduced. Fresh weight wa very much affected in the case of nitrogen-deficiency both in the shoot and in th root tissues. Dry matter accumulation, per gram fresh weight was minimum in th case of nitrogen deficiency, in which case water content was also maximur (table 1).

Ion-uptake

Anion uptake: Phosphate Deficiency of nitrogen, phosphorus and potassiun reduced the uptake of ^{32}P-phosphate by *C. arietinum* to a very large extent; th effect was most marked in case of nitrogen deficiency where the plants could tak up only about 28% of the ^{32}P taken up by the normal, nutritionally sufficient plant (table 2). This was followed by potassium and phosphorus deficiency where th uptake was curtailed by about 57%-64%. The ^{32}P-content per unit weight of shoo and root tissues also revealed largely similar trends. The ^{32}P-content of the roo tissues was always higher than that in the shoot tissues; deficiency condition however, did not alter the pattern of ^{32}P-distribution between the root and shoo tissues (table 1).

Sulphate Very similar trends were noted in the case of [^{35}S]-sulphate uptak (table 2). However, in the nitrogen-deficient plants, the roots retained a large share of the ^{35}S taken up than the non deficient, control plants, where ^{35}S wa practically evenly distributed between the shoot and the root tissues. The shoot o

Table 1. Effect of nitrogen, phosphorus and potassium-deficiency at the 1/9 level of supply on the growth of Bengal gram.

Parameter	Control	Nitrogen deficient	Phosphorus deficient	Potassium deficient
Shoot height (cm)[a]	13.65±0.01	12.19±0.10*	12.21±0.03**	11.12±0.03**
Root length (cm)	7.36±0.04	3.26±0.04**	4.16±0.02**	4.38±0.02**
Shoot : root ratio	1.54 : 1	3.73 : 1	2.93 : 1	2.53 : 1
Shoot fresh wt. (g)[a]	6.54±0.12	2.71±0.003**	2.82±0.9**	2.25±0.08**
Root fresh wt. (g)	6.31±0.09	3.07±0.12**	3.66±0.04**	3.67±0.25**
Shoot : root ratio	1.03 : 1	0.88 : 1	0.77 : 1	0.61 : 1
Shoot dry wt. (g)[a]	2.40±0.08	1.12±0.03	1.66±0.04	1.52±0.001
Root dry wt (g)	2.15±0.11	1.12±0.06**	1.45±0.002**	1.49±0.05**
Shoot : root ratio	1.11 : 1	1.00 : 1	1.14 : 1	1.02 : 1
No. of internodes	9.37±0.26	8.12±0.23*	8.12±0.29*	7.12±0.64*
No. of branches[b]	7.56±0.08	3.47±0.08**	4.86±0.05**	4.98±0.05**
No. of compund leaves	8.54±0.19	7.33±0.27	7.83±0.13**	7.20±0.36*
Leaf area (cm^2)	14.67±0.35	8.56±0.23**	7.90±0.30**	9.03±0.55**
No. of pods[b]	15.87±0.58	6.88±0.29**	13.00±1.02*	13.00±0.42**
Wt. of 1st pod (g)[b]	0.80±0.06	0.70±0.006	0.75±0.06	0.75±0.09
Wt. of 2nd pod (g)[b]	0.89±0.02	0.85±0.005	0.8±0.07	0.97±0.14
Wt. of 3rd pod (g)[b]	1.17±0.10	1.12±0.11	1.20±0.001	0.95±0.22

Plants sown on October 24, 1970.

[a] Age of plants 34 days; [b] Age of plants 68 days.

* Significant at $P=0.05$; ** Significant at $P=0.01$

Table 2. Effect of deficiency of nitrogen, phosphorus and potassium on the uptake and distribution of ^{32}P-phosphate and ^{35}S-sulphate in Bengal gram.

Nutrient level	Total cpm per plant × 10^{-6}	Shoot		Root		Shoot : Root
		Total cpm × 10^{-6}	cpm × 10^{-3} .mg^{-1} dry tissue	Total cpm × 10^{-6}	cpm × 10^{-3} .mg^{-1} dry tissue	(as per cent of total uptake)
		^{32}P-phosphate				
Control	2,773	1,083	425	1,689	637	39.0 : 61.0
Nitrogen deficient	776	216	180	560	415	27.5 : 72.5
Phosphorus deficient	1,199	494	280	705	455	41.2 : 58.8
Potassium deficient	996	337	255	659	425	33.8 : 66.2
		^{35}S-sulphate				
Control	2,009	1,042	425	968	365	51.3 : 48.7
Nitrogen deficient	614	254	212	359	252	40.7 : 59.3
Phosphorus deficient	867	476	264	390	244	59.0 : 41.0
Potassium deficient	712	388	254	324	247	54.3 : 45.7

40-Day-old plants were used.

phosphorus and potassium deficient plants received a larger fraction of th taken up than the root.

Cation uptake

Sodium Deficiency of nitrogen and potassium markedly reduced the uptak ^{24}Na, the degree of reduction being 60% and 50%, respectively. In the cas phosphorus deficiency however, the uptake was not much affected (table 3). distribution of ^{24}Na between the shoot and the root tissues was also influence deficiency conditions; in the case of phosphorus and potassium deficiency, roots retained a much larger fraction of the ^{24}Na taken up, but in the case nitrogen deficiency, opposite trends were recorded. Per unit weight of tis however, the effects of nitrogen deficiency on ^{24}Na content were detectable onl the root tissues; in the case of phosphorus deficiency, unit mass of root contai at least 40% more of ^{24}Na than in the control. The ^{24}Na content per unit weigh the shoot in the case of phosphorus and potassium deficiency was decreased 50% or more (table 3).

Potassium The effect of nitrogen deficiency on ^{42}K uptake was most strik where the reduction was of the order of 40% (table 3). In the control, the ^{42}K tak up was practically equally distributed between the shoot and root tissues; under deficiency conditions, the shoot received 3 to 6 times the amount presen the root tissues. In phosphorus and potassium deficient plants, the sh possessed 46 to 78%, more of ^{42}K than in the non-deficient plants. The ^{42}K cont per unit tissue weight however, was only twice the amount detected in the cont On per unit weight basis, the root tissues, particularly in the case of potassi deficient plants contained 65% less of ^{42}K. Nitrogen and phosphorus deficien reduced the potassium content per unit weight of root tissue to the extent of 35% both cases.

Calcium The deficiency of nitrogen reduced the uptake of ^{45}Ca to the larg extent, the amount taken up was even less than one-half of the nutritiona sufficient plants. In the case of phosphorus and potassium deficiency, uptake w reduced to about two-thirds. The shoot was more affected than the root, t former possessing only one-third of the total ^{45}Ca taken up (table 3). T distribution of ^{45}Ca per unit weight of shoot and root tissues, however, w decreased only to a small extent.

Manganese ^{54}Mn uptake was curtailed by more than 50% by the deficiency each of the three elements studied; here also the effect was most prominent in t case of nitrogen deficiency (table 3), where the curtailment was of the order of 70 ^{54}Mn taken up by the non-deficient plants was distributed between the shoot a the root tissue in the ratio of 2:3; but in the nitrogen deficient plants, the shoot a root shared almost equally the ^{54}Mn taken up. The root possessed only 25% to 45 of the ^{54}Mn found in the non-deficient control. The ^{54}Mn content per unit weight shoot and root tissues was least in the case of phosphorus deficiency.

Zinc Nitrogen deficiency also reduced the uptake of ^{65}Zn most; this was true f both shoot and root tissues (table 3). In the case of phosphorus and potassiu

Table 3. Effect of deficiency of nitrogen, phosphorus and potassium on the uptake and distribution of ^{24}Na, ^{42}K, ^{45}Ca, ^{54}Mn, ^{65}Zn and ^{59}Fe in Bengal gram.

Nutrient level	Total radioactivity (cpm per plant (cpm per plant × 10^{-3})	Shoot Total cpm × 10^{-3}	Shoot cpm mg^{-1} dry tissue	Root Total cpm × 10^{-3}	Root cpm mg^{-1} dry tissue	Shoot : Root (As per cent of total radioactivity)
^{24}Na						
Control	4,891	2,168	903	2,723	1,112	43.3 : 57.8
Nitrogen deficient	2,045	1,173	903	870	678	57.4 : 42.6
Phosphorus deficient	4,113	1,085	594	3,027	1,593	26.3 : 73.7
Potassium deficient	2,457	805	512	1,645	1,044	32.8 : 67.2
^{42}K						
Control	2,052	988	426	1,064	692	45.1 : 55.9
Nitrogen deficient	1,252	947	902	305	453	75.6 : 24.4
Phosphorus deficient	1,919	1,449	827	470	448	75.4 : 24.6
Potassium deficient	2,029	1,754	1,110	264	240	86.6 : 13.4
^{65}Ca						
Control	2,493	1,050	433	1,443	750	42.1 : 57.9
Nitrogen deficient	1,142	421	401	721	687	36.8 : 63.2
Phosphorus deficient	1,649	561	365	1,088	702	34.0 : 66.0
Potassium deficient	1,647	592	382	1,054	691	35.0 : 64.1
^{54}Mn						
Control	2,684	1,030	429	1,653	798	38.3 : 61.7
Nitrogen deficient	805	377	355	428	408	53.4 : 46.6
Phosphorus deficient	982	445	287	536	346	45.3 : 54.7
Potassium deficient	1,314	564	371	750	419	42.9 : 57.1
^{65}Zn						
Control	1,282	572	236	710	406	45.3 ; 54.7
Nitrogen deficient	577	215	205	362	343	37.2 : 62.8
Phosphorus deficient	818	280	182	532	343	34.9 : 65.1
Potassium deficient	815	320	207	494	324	39.3 : 61.7
^{59}Fe						
Control	2,786	1,462	631	1,323	680	52.5 : 47.5
Nitrogen deficient	1,418	743	563	675	509	52.4 : 47.6
Phosphorus deficient	1,832	859	522	973	618	46.8 : 53.2
Potassium deficient	1,534	705	432	829	525	45.9 : 54.1

37-Day-old plants were used.

deficiency, the plants suffered almost to the same extent. Irrespective of deficiency conditions, the roots possessed more of ^{65}Zn than the shoot tissues.

Iron The uptake of ^{59}Fe was almost equally affected by nitrogen and potassium deficiency (table 3). In the phosphorus deficient plants, the roots contained the largest amount of ^{59}Fe. The ^{59}Fe content per unit weight of shoot tissues was least in the case of potassium deficiency and highest among the deficient plants in the case of phosphorus deficient roots.

Mobilization of ions

The ^{32}P taken up by Bengal gram, as in the case of pea (Biswas and Sen, 1959) was first transported to the apical region and then moved downwards into the lower leaves, older leaves receiving smaller amounts. Deficiency conditions influenced the distribution pattern more strikingly when the plants had flowered and fruited.

^{32}P was found to accumulate largely in the apical bud and the young leaves, irrespective of deficiency conditions; when the plants flowered, the accumulation of ^{32}P in the first pod which was the youngest, however, was reduced considerably under deficiency conditions (table 4). The severity of the effects was much less in

Table 4. Effect of deficiency of nitrogen, phosphorus and potassium on the distribution of [^{32}P]-phosphate and [^{35}S]-sulphate in the pods and adjacent leaves of Bengal gram.

Organs	Control	Nitrogen deficient	Phosphorus deficient	Potassium deficient
		[^{32}P]-phosphate		
1st pod				
Total radioactivity (cpm×10^{-3})	101	72	78	62
cpm/ mg dry tissue	145	120	121	104
Leaf subtending 1st pod				
Total radioactivity (cpm×10^{-3})	84	78	61	69
cpm/mg dry tissue	120	104	103	99
2nd pod				
Total radioactivity (cpm×10^{-3})	91	88	54	56
cpm/ mg dry tissue	102	98	78	71
Leaf subtending 2nd pod				
Total radioactivity (cpm×10^{-3})	65	59	55	49
cpm/mg dry tissue	82	78	79	82
3rd pod				
Total radioactivity (cpm×10^{-3})	86	84	74	76
cpm/mg dry tissue	72	70	62	64
Leaf subtending 3rd pod				
Total radioactivity (cpm×10^{-3})	54	36	41	44
cpm/mg dry tissue	62	52	69	63
		[^{35}S]-sulphate		
1st pod				
Total radioactivity (cpm.×10^{-3})	108	80	88	97
cpm/mg dry tissue	120	100	104	108

Organs	Control	Nitrogen deficient	Phosphorus deficient	Potassium deficient
Leaf subtending 1st pod				
Total radioactivity (cpm×10^{-3})	97	73	61	49
cpm/mg dry tissue	108	92	82	71
2nd pod				
Total radioactivity (cpm×10^{-3})	91	78	80	107
cpm/mg dry tissue	102	98	92	93
Leaf subtending 2nd pod				
Total radioactivity (cpm×10^{-3})	82	61	57	51
cpm/mg dry tissue	92	82	74	69
3rd pod				
Total radioactivity (cpm×10^{-3})	89	86	97	84
cpm/mg dry tissue	78	82	81	71
Leaf subtending 3rd pod				
Total radioactivity (cpm×10^{-3})	58	43	45	36
cpm/mg dry tissue	71	62	51	52

68-Day-old plants were used.

the third pod. This distribution pattern was, however, not correlated with that observed in the leaves subtending the pods. Thus nitrogen-deficiency effects were more marked in the leaves subtending the lower and the more aged pods than the leaves subtending the first pod. Phosphorus deficiency had almost similar effects in the case of the leaves subtending the first and the third pods. On per unit weight basis, the effects of deficiency were much less prominent; in some cases, no sign of deleterious effect was discernible.

In the normal plants, ^{35}S was well mobilized. The deficient plants had a relatively slow rate of uptake and mobilization of ^{35}S. But the shoot apices and the young leaves invariably retained a larger share. Deficiency effects on mobilization of ^{35}S in the fruited plants were not as prominent as in the case of ^{32}P. The ^{35}S-content of the pods was curtailed by no more than 25%. The effect on the first pod, although not very pronounced was the most marked. In the subtending deficient leaves, however, ^{35}S content was reduced in all cases, the effect of potassium deficiency being most prominent.

Deficiency of nitrogen did not help the distribution of ^{24}Na in all the leaves. The phosphorus and potassium deficient apical regions accumulated more of ^{24}Na. Old leaves situated near the base of the plants were only slightly radioactive. ^{42}K did not reach the leaflets of the lower leaves during the three-hour uptake. The distribution patterns in phosphorus and potassium deficient plants did not significantly differ from that of control plants. Much of ^{45}Ca taken up was located in the stem and the apical region in the control and deficient plants.

Phosphorus deficiency improved the mobilization of ^{54}Mn, but mobilization was reduced considerably in the case of potassium deficiency. Mobilization of ^{65}Zn was also vastly improved under phosphorus deficiency. Accumulation of ^{65}Zn in the apical region was not affected by any deficiency condition.

The mobilization of iron was improved under deficiency of potassium, although the amount of ^{59}Fe taken up was less than that of the control. The potassium and phosphorus deficient plants accumulated a much larger amount of ^{59}Fe in the apical regions than in the control.

Discussion

It has been observed that except in the case of potassium, the uptake of all the ions studied was decreased in the plants deficient in nitrogen, phosphorus and potassium. This reduced uptake may be due to: (i) decreased surface area of the root system, (ii) reduced leaf area, (iii) reduced availability of metabolic energy for ion uptake and (iv) partial inhibition of the synthesis of the carriers required for transport of ions across membranes or their structural alteration resulting in an impairment of functional efficiency. The marked reduction in root length (table 1) would result in a decreased surface area of the root system. The leaf area was reduced by about 40% or more; this would result not only in reduced photosynthesis but also in decreased ion uptake, since the effective area of evaporation per plant was also reduced. Although very little is known about deficiency effects on transpiration, it may be of interest to mention here that Atkinson and Davison (1972) observed a decrease in the rate of transpiration due to phosphorus deficiency. According to Graham and Ulrich (1972), permeability of root to water is greatly decreased in potassium deficiency. Das (1974) and Das and Sen (1974) observed an inhibition of respiration and photosynthesis, particularly due to deficiency of nitrogen and phosphorus; this would reduce the availability of metabolic energy for ion uptake. Das (1974) and Das and Sen's (1971) observation that nucleic acid and protein synthesis is inhibited by deficiency conditions may suggest that the synthesis of protein-type carriers required for ion-uptake may be inhibited by mineral deficiency. Uptake of all the elements studied were most severely affected by deficiency of nitrogen; the effect of phosphorus deficiency was least. Potassium deficiency reduced Na and Ca uptake by 50% and 30%, respectively. In maize also, potassium deficiency reduced Ca-uptake (Singh, 1970) but in sugar beet it increases Na concentration soon after cut off (Terry and Ulrich, 1970). Similar results were also observed in rice (Das and Sen, 1980) and wheat (Das, 1974).

Deficiency conditions also exercised pronounced influence on the distribution of ion taken up between shoot and root tissues and certain types of deficiency completely altered the pattern as in the case of distribution of sulpahte, Na and Mn by nitrogen deficiency and of potassium by all the three deficiency conditions. Different regions of a plant vary in their capacity for absorption and transport of ions and this difference plays an important role in determining the distribution of ions between the various parts of a plant (Higinbotham *et al.,* 1962; Scott and Martin, 1962).

As in the case of pea (Biswas and Sen, 1959), the ions taken up first move to the top most part of the plant where the youngest leaves and buds are situated and then move downwards. When the apices and buds are transformed into flower, there is an accumulation of nutrients in the flowers and fruits and in the leaves which subtend them, irrespective of deficiency condition.

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Response of blood glucose, hepatic glycogen and pancreatic islets to treatment of sulphydryl inhibitors in *Clarias batrachus* L.*

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Abstract. The correlative effects of the two sulphydryl inhibitors viz., the mono-and dithiol reagent, alloxan and cobalt chloride, were studied in the fresh water air breathing catfish, *Clarias batrachus*. Alloxan induced a typical mammalian-like triphasic response and necrobiotic changes in the islet β-cells. Cobalt chloride elicited a discontinuous hyperglycemia with cytopathological changes in β-cells following nearly the same sequence as those induced by alloxan. However, the α-cells of cobalt-treated fish showed, unlike those after alloxanization, specific degranulation, vacuolization and nuclear enlargement. The changes in the hepatic glycogen content of two experimental groups were observed to be equal. Both the drugs appeared to be detrimental to the normal islet function and were, thus, overtly or potentially diabetogenic agents to the fish.

Keywords. *Clarias batrachus;* islet cells; histophysiology; alloxan; cobalt chloride.

Introduction

The use of sulphydryl inhibitors in mammals has gained considerable interest in speculations concerning the pathogenesis and etiology of diabetes mellitus (Dunn *et al.*, 1943; Hultquist, 1959; Boquist, 1967, 1968; Havu, 1969). Our knowledge of the role of these substances in impairing the normal histology of the Langerhans' islets and the blood glucose homeostasis in lower vertebrates, including fish, is scanty and undeducible (Miller and Wurster, 1958; Falkmer, 1961; Rangnekar and Sabnis, 1967; Chavin and Young, 1970; Khanna and Gill, 1975; Kumar and Khanna, 1978). Teleosts with the islet organ as a grossly visible 'Brokmann corpuscle' offer unequable possibilities for chemical and metabolic studies of pure islet tissue (Falkmer, 1961). The similarities of the structural patterns of teleostean and mammalian islet tissues make possible extrapolation to human tissues, of experimental results obtained from the lower vertebrates. The present study was undertaken to make detailed observations on the effects of alloxan and cobalt chloride, on the plasma glucose level, hepatic glycogen content and on the light microscopical picture of the pancreatic islets of a freshwater catfish, *Clarias batrachus.*

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Materials and methods

Adult specimens of both the sexes of *Clarias batrachus* procured from a commercial source and acclimated to laboratory conditions were used. The weight and length of the test animals varied between 60-125 g and 12-20 cm respectively. The photoperiod and temperature although not rigorously controlled, were of 11 h, L (light): 13 h, D(dark) regime and 19-25°C, respectively. Freshly prepared 5% (w/v) and 0.5% (w/v) solutions respectively of alloxan (Hexahydrate, Merck,Germany) in phosphate-citrate buffer; pH 4 and cobalt chloride (in 0.9% NaCl) were administered deep into the epaxial musculature in approximately 350 specimens arranged into the following 6 groups, each with atleast 40 individuals:

I Fish receiving alloxan at a dose of 200 mg/kg body weight;

II Fish receiving alloxan at double the previous dose, i.e., 400 mg/kg body weight;

III A control group receiving phosphate-citrate buffer at the same w/v ratio;

IV A group for testing the effect of cobalt chloride (25 mg/kg body weight;

V A group administered cobalt chloride at double the previous dose, i.e., 50 mg/kg body weight; and

VI Control groups which received the vehicle solution at the same w/v ratio.

The LD (lethal dose) 50 value for alloxan and cobalt chloride were found to be 380 mg/kg and 90 mg/kg respectively. From each experimental and control group, 3 to 5 individuals were sacrificed at the predetermined intervals ranging between 1/2 h and 240 h. During autopsy, blood samples (0.1 ml) and small pieces of liver (200-300 mg) were collected and processed for the estimation of plasma glucose (mg%) and liver glycogen (mg/g) following the method of Nelson (1944) and Zarrow *et al.* (1964) respectively. For cytopathological study, the pancreatic islets were fixed in Bouins' or Helly's fluids and sectioned into 4-6 nm slices. Serial sections were stained, after usual deparaffinization and dehydration by the aldehyde fuchsin (AF) (Maske, 1955) and phosphotungstic acid haematoxyline (PTAH) (Hultquist and Tagner, 1949) techniques. All the test animals were starved throughout the experimental period.

Results

Blood glucose

The blood glucose of 12 normal, untreated specimens of *C. batrachus* averaged 60.23 ±3.83 mg%. Glycovariations resulting from the administration of various doses of alloxan in the fish are depicted in figure 1. Initially, a lower dose of alloxan (200 mg/kg) could not produce any appreciable change in the blood glucose level, which remained in close proximity of the glycemic limits of control animals. an elevation in its level was however evident 5 h after the injection (86.5 ±5.83 mg%) and this continued further to 117 ±4.27 mg%. A significant drop in glycemia was recorded 72 h after the administration resulting in a hypoglycemic state (mean, 43.5 ±6.34 mg%). A further increase in the blood glucose, although of a lower magnitude as compared to the initial one, was registered during the next

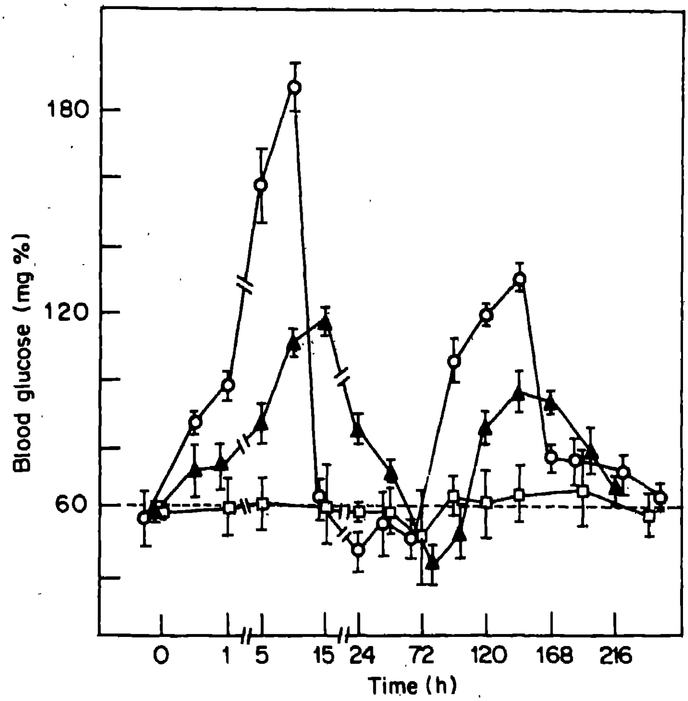

Figure 1. Changes in blood glucose (mg%) in *Clarias batrachus* following treatment with alloxan. Designations: (□), control: (▲), 200 mg/kg: (O), 400 mg/kg. The horizontal line denotes mean of 120 untreated fish. The vertical bars represent two standard errors of the mean. Each point represents mean of 3-5 individual determinations.

48 h, and the fish became normo-glycemic in 8 days. Subsequent to alloxan injection, at a dose of 400 mg/kg body weight, an increase in glycemia occured as early as 1/2 h, reaching its peak, 187 ±7.34 mg%, 5 h earlier than for the previous dose. The hyperglycemic phase was recorded 48 h earlier as compared to the lower dose. The second hyperglycemia was comparable to the initial increase in the blood glucose induced by a dose of 200 mg/kg and the fish became normoglycemic within the same number of days (figure 1).

A rise in blood glucose level (81 ±2.95 mg%) was recorded 1 h after injection of cobalt chloride at a dose of 25 mg/kg body weight. It was followed by a sharp decline leading to a transitory return to the normal levels. There was a second increase in blood glucose level 24 h post-treatment (96.6 ±5.5 mg%) and the highest values were evoked after 48 h (116 ±3.46 mg%). Thereafter, the glucose level showed a tendency to decrease. A higher dose, 50 mg/kg body weight induced sharp changes in the glycemia. A hyperglycemia observed immediately after the administration was followed up by an intense decline, leading to a hypoglycemic condition (47 ±2.5 mg%), 15 h after the treatment. The glucose content of blood rose again in the same observation period as with the previous dose (102.7 ±9.31 mg%) and continued to do so reaching the peak level (130 ±8.5 mg%), 73 h post-treatment. Four days after the administration, the blood glucose of one oi 4 fish sacrificed reached the normal values while the other 3 were still mark hyperglycemic. Till the 7th day of the treatment, the blood glucose level distinctly higher (figure 2).

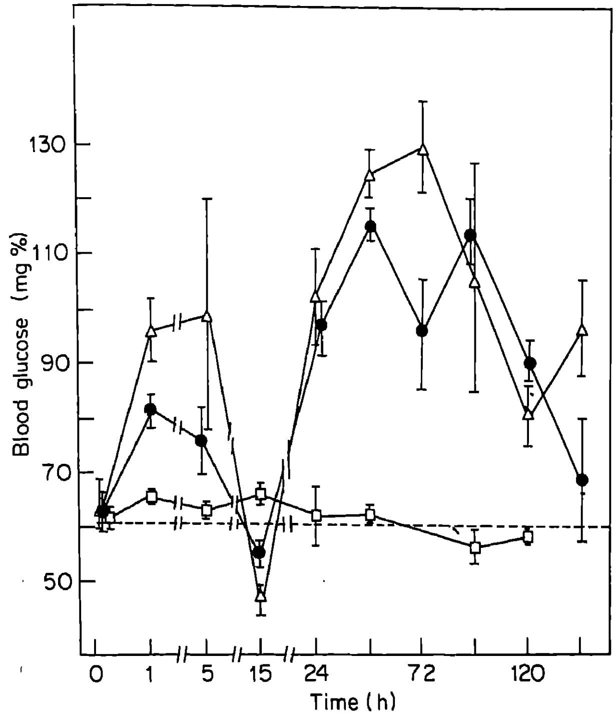

Figure 2. Changes in blood glucose (mg%) in *C. batrachus* after treatment with col chloride. Designations: (●),25 mg/kg: (Δ),50 mg/kg, other designations the same as in figu

Liver glycogen

A decrease in the amount of hepatic glycogen was observed within 24 h of alloxa zation, when the values fell from 58.6 ±3.3 mg/g to 38.8 ±0.5 mg/g, and the valu remained significantly below the control range till 120 h. An appreciable loss glycogen stores of liver was recorded within 5 h (49.5 ±3.04 mg/g) of treatm with alloxan at a higher dose, and was reduced further to a low level of 29.5 ±4 mg/g after 24 h. Normal values were, however, restored within 8 days (figure 3).

The hepatic glycogen also responded to cobalt salts, with a fall in its conte beginning after 1.5 h (47.9 ±3.0 mg/g). The effect was more significant after 2 reaching a low level of 34.3 ±2.24 mg/g, 48 h after treatment of cobalt chloride (mg/kg). However, only mild changes in the liver glycogen content were record between 72 and 96 h. The higher dose, 50 mg/kg, elicited a fall in liver glycog about 10 h earlier than the previous dose and a nadir value of 23.3 ±1.66 mg/g w registered 24 h post injection. Till the end of the experimental period, a norm amount remained unrecuperated (figure 4).

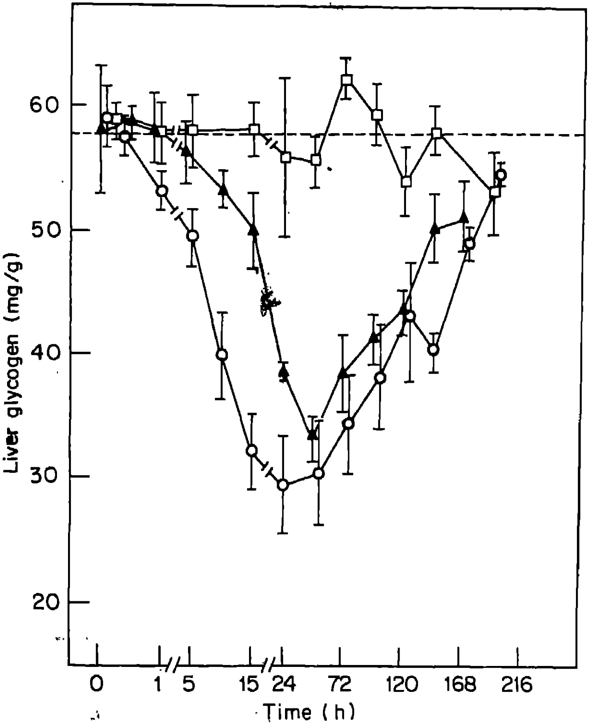

Figure 3. Changes in liver glycogen (mg/g) in alloxanized *C. batrachus*. Designations the same as in figure 1.

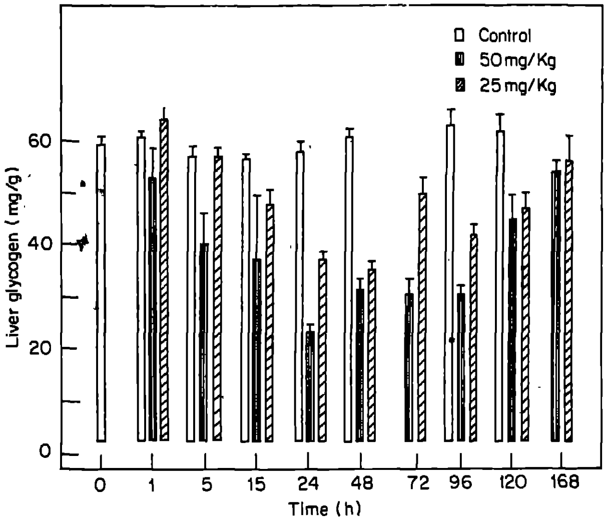

Figure 4. Modifications in liver glycogen (mg/g) in *C. batrachus*, treated with cobalt chloride. Legends same as in figure 2.

Histology of the islets

Light microscopically, the α_1 (argyrophilic), α_2 (nonorgyprophilic) and β-cells (aldehyde fuchsinophilic) make up the islets (figure 5) the approximate ratio between α and β cells being 40:60. The necrotizing effects of alloxan and cobalt chloride on the pancreatic islets have been shown in figures 5 to 9 and 10-13 respectively.

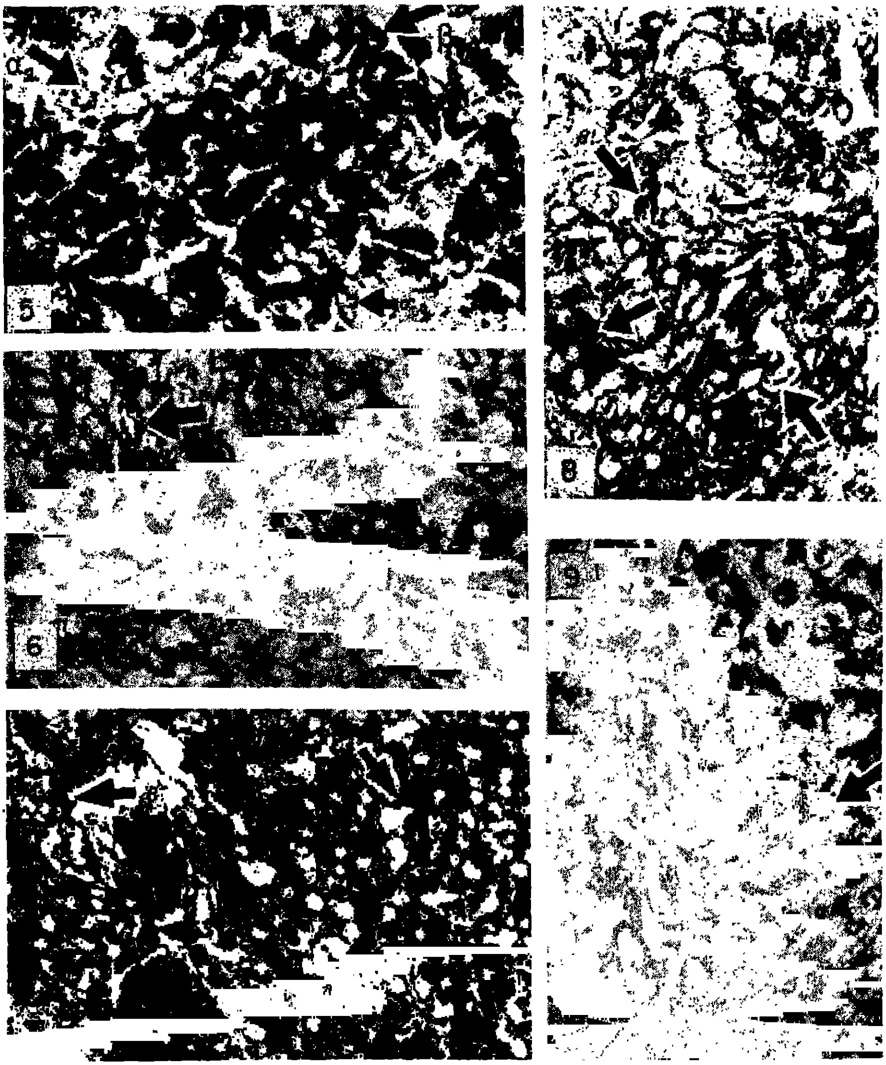

Figure 5-9. Photomicrographs of the principal islets of alloxan-treated *C-batrachus*. ×400. AF stain except Figure 7. **5.** Control showing normal α and β -cells. AF-light green stain. **6.** and **7.** 200 mg/kg alloxan dose. Arrow indicates changes in β-cells. **6.** After 10 h. **7.** After 120 h. **8.** and **9.** 400 mg/kg dose of alloxan. Changes in the cellular elements are indicated by arrows. **8.** After 5 h. **9.** After 24 h, PTAH stain.

Alloxan administration at a dose of 200 mg/kg body weight evoked mild degranulation of β-cells within 1/2 h. The damage to the β-cells was more conspicuous by about 10 h after the injection and widespread necrosis of both α and β-cells was apparent (figure 6). Clumping and atrophy of β-cells were prominently visible until 120 h after alloxanization (figure 7). The extent of cellular damage was more in the islets of the fish at a dose of 400 mg/kg alloxan and significant alterations in the β-cell configuration were observed in the islets studied within 5 h. Here, only a few sparsely granulated β-cells were visible and the rest became necrotic and shrunken resulting in the formation of large empty spaces (figure 8). At this stage, some of the islets counterstained with light green, showed vacuolized α-cells. A PTAH-stained preparation showed loss of cytoplasmic granules in the α-cells, 24 h post-injection (figure 9). The material examined close to the end of the experimental period revealed depletion of the beta granules.

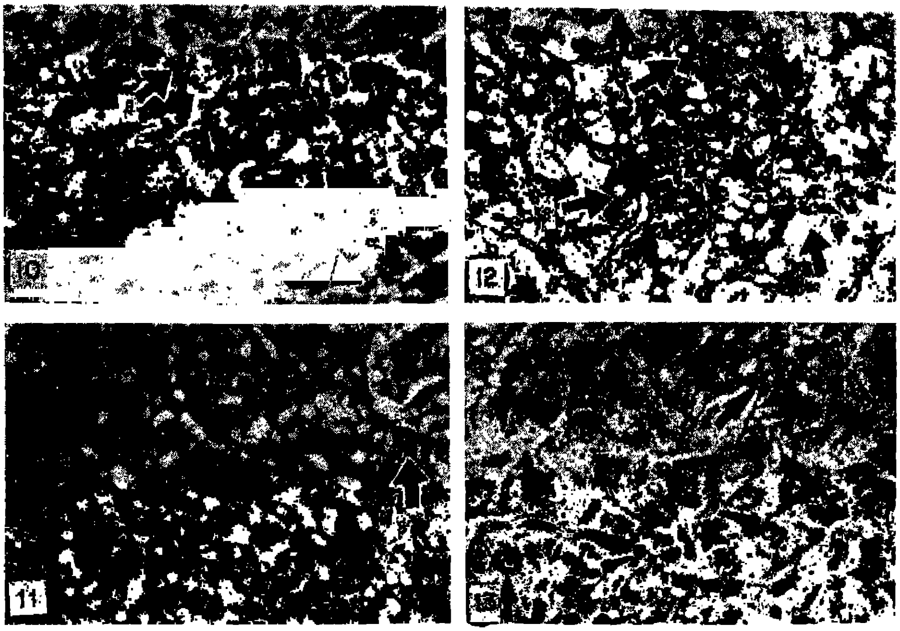

Figure 10-13. Photomicrographs of the principal islets of *C.batrachus* treated with cobalt ×400. AF stain except figure 13. Arrow indicates changes in the α-and β-cells. **10**, and **11**. After treatment with 24 mg/kg dose. **10**. After 5 h, **11**. After 24 h. **12** and **13**. After 50 mg/kg dose of cobalt chloride. **12**. After 1 h. **13**. After 48 h PTAH staining.

The cobalt salt caused specific damage to α-cells while the β-cells underwent histological changes that were nearly similar to those seen after alloxanization. The lower dose (25 mg/kg) produced moderate degranulation of β-cells and degeneration of α-cells leaving intercellular spaces (figure 10). The islets examined between 5 h and 48 h after the treatment showed varying degrees of degeneration of β-cells. The histological effect of this dose was not uniform even in the same islet, thus perfectly normal as well as damaged cellular elements were observed (figure 11). At a higher dose, 50 mg/kg, the changes in the α and β-cells were apparently uniform, rapid and more pronounced. Considerable loss of cytoplasmic stainability, clumping and atrophy of β-cells were observed in an islet within 1 h of treatment of cobalt chloride (figure 12); some of the α-cells also presented vacuolisation. Fish autopsied 15 h after the treatment had their islets exhaustively damaged. Hypertrophy of the α-cells was clearly visible in a PTAH-stained section of the islet at 48 h (figure 13). Depletion of granules and mild vacuolization of α-cells was revealed until 120 h post-treatment.

An increase, albeit slight, was found in the blood glucose level of buffer injected controls only; the pancreatic islets otherwise remained perfectly normal.

Discussion

Alloxan, although well known for causing selective β-cell destruction and consequent hyperglycemia in several vertebrates (Rangnekar and Sabnis, 1967; Boquist, 1968; Kumar and Khanna, 1978) does not exhibit a uniformity in its effects. The present study has revealed that *Clarias batrachus* responds to alloxan treatment and the characteristic triphasic response of mammals is followed in the fish. Thus, unlike a monophasic hyperglycemia reported in most of the alloxan-treated teleosts (*Cottus scorpius,* Falkmer, 1961; *Heteropneustes fossilis,* Khanna and Rekhari, 1972; *Channa punctatus,* Gill, 1974), and other poikilotherms (*Rana tigrina,* Kumar and Khanna, 1978), the present findings are consistent with earlier reports on mammals and the teleost, *Carassius auratus* (Chavin and Young, 1970). However, in contrast to the observations made in the alloxanized gold fish, the hypoglycemic phase in *C. batrachus* was considerably delayed up to 24-72 h, as also in *Scorpaenea scropha* (Saviano, 1947). In the present experiments, the fish revealed dose-dependent modifications with the initial hyperglycemia being of relatively higher magnitude as compared to the second one. Unlike mammals, none of the injected fish became permanently diabetic. The hepatic glycogen content, on which virtually no study has yet been attempted, registered a monophasic depletion in the catfish under study.

Like alloxan, administration of cobalt salts to experimental animals has been found to produce variations in the blood glucose level (Hultquist, 1959; Boquist, 1967, Havu, 1969; Calugareanu, 1970: Miller and Wuster, 1958). *C. batrachus* takes the same time as some other teleosts (Falkmer, 1961; Rekhari, 1970; Khanna and Gill, 1975), in exhibiting hyperglycemic response to the injection of cobalt salt. An initial hyperglycemia manifested immediately after the treatment, was followed by a transitory lowering of blood glucose. The glycemia advanced again and the peak response of the drug was noticed between 48 and 72 h after which the blood glucose registered a decreasing tendency although the values were markedly higher until the 7th day of the treatment.

Changes in blood glucose level following treatment with alloxan and cobalt salts have been correlated by some authors with the degenerative alterations occuring concomittantly in the pancreatic islets. The cytotoxic nature of alloxan has been well documented, atleast in mammals (Dunn *et al.*, 1943; Boquist, 1968). In contrast, the teleosts have been found to react differently from mammals to alloxan treatment; while the islets of some fishes remain unaffected (Pallot and Schatzle, 1953), others exhibit a wide variety of cytological lesions in their β-cells (Falkmer, 1961; Falkmer and Olsson, 1962; Chavin and Young, 1970; Khanna and Rekhari, 1972; Gill and Khanna, 1974). In common with many of these teleosts, alloxan treatment in *C. batrachus* elicited changes, from moderate to extensive, in the islet β-cells. Depletion of cytoplasmic granules was also observed in the α-cells, probably due to loss of cohesion in the cell cords. Differences in the response of individual catfish were however noticed even with the same alloxan dose. It is interesting to note that islets of many specimens with significant fluctuations in the glycemia and glycogen levels remained perfectly normal probably due to inactivation and uneven distribution of the injured alloxan which might have failed to reach all the islets quickly due to a single circuit circulatory system. These islets probably continued to function normally and, perhaps, this accounted for the inability of the fish to become permanently alloxan-diabetic.

While some fishers, viz., *Opsanus tau* (Fodden, 1956) and *Scorpaena scropha* (Mosca, 1957) have registered their insensitivity to cobalt treatment in sofar as histological changes in the pancreatic islets are concerned, others such as *C. scorpius* (Falkmer, 1961), *H. fossilis* (Rekhari, 1970) and *C. punctatus* (Khanna and Gill, 1975) accorded with mild to widespread degenerative changes in both α and β-cells. The histological changes observed in the pancreatic islets of *C. batrachus* are much the same as reported in the above mentioned fishes, which consisted of slight degranulation of both α and β-cells initially and widespread damage of the cellular elements at a later stage of the experiment.

The mechanism by which SH-inhibitors exert the diabetogenic effect has been discussed, and the importance of blood glutathione content and the amount of zinc in islet β-cells emphasized (Falkmer *et al.*, 1964). These substances possess the property to crystallize insulin with the same high efficiency as zinc, and the β-cells which manufacture and store insulin (Landall *et al.*, 1965), tend to absorb the circulating metals from the blood. The absorbed metals combine with SH-group containing substances leading to metabolic derangements in the β-cell membranes and destroy their permeability, promoting thereby, the resultant leakage of insulin (Cooperstein *et al.*, 1964; Watkins *et al.*, 1973). Therefore, the SH-inhibitors appear to exert their direct action on the β-cells and not through the medium of disturbed blood glucose level, and this probably accounts for the absence of a synchrony between the changes in glycemia and the islet histology of *C. batrachus.*

Apart from the fact that both drugs can evoke cell necrosis and apparent decrease in the islet zinc content, the doses seem to be of importance for the effect as regards the β-cell necrotizing potency. It may be contended from the foregoing discussion that though both the SH-inhibitors evoke changes in glycemia and hepatic glycogen content and also cause necrobiosis of the cellular elements of the pancreatic islets, the histophysiological effects of cobalt are not quite as high as after alloxanization. The response of the fish to SH-inhibitors presents a condition somewhat akin to that in mammals. The fish may therefore be taken as an experimental model for the study based on the characterization of the β-cell function in lower vertebrates.

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Cytokinin binding proteins from mammalian sera

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Abstract. A cytokinin-binding protein fraction was isolated from normal rabbit sera by affinity chromatography. The protein fraction bound tritium labelled N^6-(Δ^2-isopentenyl) adenosine and the order of inhibition of this binding by competing non-radioactive compounds was, N^6-(Δ^2-isopentenyl) adenosine >N^6-benzyladenosine >zeatin-riboside >N^6-(Δ^2-isopentenyl) adenine >kinetin riboside >adenosine. The protein fraction showed broad specificity, the prefered cytokinin being N^6-(Δ^2-isopentenyl) adenosine. This is the first report of the isolation of cytokinin binding proteins from mammalian sources.

Keywords. N^6-(Δ^2-isopentenyl) adenosine; binding protein; rabbit sera.

Introduction

Cytokinins promote cell division in plants (Miller, 1963; Letham, 1967). Cytokinins occur in plants in the free form and as components of their tRNAs. Receptor proteins for cytokinins and other phytohormones are present in plant tissues (Jayabaskaran, 1981; Dodds and Hall, 1980).

There is no evidence in literature that cytokinins occur free in mammalian systems or that they have any function in these systems. But cytokinins are known to bring about a variety of perturbations in mammalian cells. Thus, N^6-(Δ^2-isopentenyl) adenosine inhibits growth of leukaemic cells *in vivo* (Suk *et al.*, 1970) and *in vitro* (Rathbone *et al.*, 1972). Gallo *et al.* (1969) observed that N^6-(Δ^2-isopentenyl) adenosine inhibits mitosis of lymphocytes stimulated by phytohemagglutinin, at a concentration of 1 μM. Quesney-Huneeus *et al.* (1980) have shown that N^6-(Δ^2-isopentenyl) adenine and zeatin can replace mevalonate to restore DNA replication in compactin blocked baby hamster kidney cells, but that N^6-(Δ^2-isopentenyl)adenosine is not effective.

In animal systems, cytokinins have been detected only in tRNAs (Letham, 1978; Senapathy, 1978). It is known that the presence of N^6-(Δ^2-isopentenyl)adenosine in certain species of tRNAs is essential for the proper function of those tRNAs in

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Abbreviations used: i^6A/i^6 Ado, N^6-(Δ^2-isopentenyl) adenosine; pi^6Ap, N^6-(Δ^2-isopentenyl) adenosine 5',3'(2') diphosphate; pi^6Ap>, 5'-phosphoryl-N^6-(Δ^2-isopentenyl) adenosine 3'-2' cyclic phosphate; EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; AH-Sepharose, Aminohexyl Sepharose; TBS, Tris-buffered saline (0.01 M Tris-HCl, pH 7.5 containing 0.14 M NaCl and 0.02% NaN_3); TBHS, Tris-buffered high concentration saline (0.01 M Tris-HCl, pH 7.5 containing 0.64 M NaCl and 0.02% NaN_3).

protein synthesis, probably in their binding to ribosomes. In a preliminary communication Fox and Erion (1975) have reported the presence of cytokinin binding sites on rat liver ribosomes.

When N^6-(Δ^2-isopentenyl)adenosine antibodies were purified in our laboratory on an AH-Sepharose-pi^{6}A$_p$>column or an i^{6}Ado-AH-Sepharose column (Senapathy, 1978; Jayabaskaran, 1981) and the purified antibodies were subjected to gel electrophoresis, two protein bands, in addition to IgG, were observed. It was suspected that these could be N^6-(Δ^2-isopentenyl) adenosine-binding proteins normally present in rabbit sera, and attempts were made to isolate them. This paper reports the affinity chromatographic isolation of a cytokinin binding protein fraction from normal rabbit sera.

Materials and methods

Serum was prepared from rabbits obtained from the local market. N^6-(Δ^2-isopentenyl)adenosine, N^6-(Δ^2-isopentenyl)adenine, zeatinriboside, N^6-benzyladenosine, kinetinriboside, adenosine, guanosine, mevalonic acid, AH-Sepharose and bovine serum albumin were from Sigma Chemical Co., St. Louis, Missouri, USA. Nitrocellulose filters (0.45 µ, MDI filters) were from microdevices, Ambala. Tritium labelled N^6-(Δ^2-isopentenyl)adenosine was prepared at the Bhabha Atomic Research Center, Bombay, by the Wilzbach procedure (1957), by exposing N^6-(Δ^2-isopentenyl)adenosine to tritium gas. It was purified by chromatography on Sephadex LH-20 (Armstrong *et al.*, 1969). The specific activity of the ^{3}H-i^{6}A was 56,275 cpm/pmol.

Preparation of N^6-(Δ^2-isopentenyl) adenosine 5′,3′(2′) diphosphate

The synthesis was done by the phosphorylation of N^6-(Δ^2-isopentenyl) adenosine with cyanoethyl phosphate in the presence of dicyclohexyl carbodiimide and the subsequent removal of the cyanoethyl group under alkaline conditions, according to the procedure of Humayun (1974).

Preparation of the affinity column

The 5′-phosphate group of N^6-(Δ^2-isopentenyl)adenosine 5′,3′(2′) diphosphate was coupled to the amino groups in AH-Sepharose using EDC as the condensing agent, according to the procedure used by Humayun (1974) for coupling the same diphosphate to bovine serum albumin. Humayun has shown that on EDC treatment the 5′,3′(2′) diphosphate cyclises to give 2′-3′ cyclic phosphate leaving the 5′ phosphate for condensation to the amino groups. The amount of pi^{6}Ap coupled to AH-Sepharose was spectroscopically determined after solubilization by NaOH and sodium borohydride treatments (Failla and Santi, 1973) and was found to be 2.7 µmol per ml of packed volume of AH-Sepharose.

Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

For analyzing the specificity of the binding of ^{3}H-i^{6}A to the isolated protein fraction, the nitrocellulose filter assay was carried out as described by Humayun and Jacob (1973) for antibody and hapten systems.

Results

Affinity chromatography

AH-Sepharose-pi^{6}Ap> column (0.4×3.5 cm) was equilibrated with TBHS (0.01M Tris-HCl, pH 7.5 containing 0.64M NaCl). Fifty ml of normal rabbit sera was made up to 150 ml with concentrated buffer so that the final buffer composition was the same as that of the equilibrating buffer and then loaded onto the column at a flow rate of 3 ml/h. The column was first washed with TBHS (200 ml) and then eluted with 5mM i^{6}A in TBHS. One ml fractions were collected and dialysed separately against TBS (0.01M Tris-HCl, pH 7.5 containing 0.14M NaCl). Protein in each fraction was estimated by Lowry's method. The elution profile is shown in figure 1. There is only one protein peak (895 µg). Fractions were pooled, concentrated and dialysed extensively in TBS to remove i^{6}A.

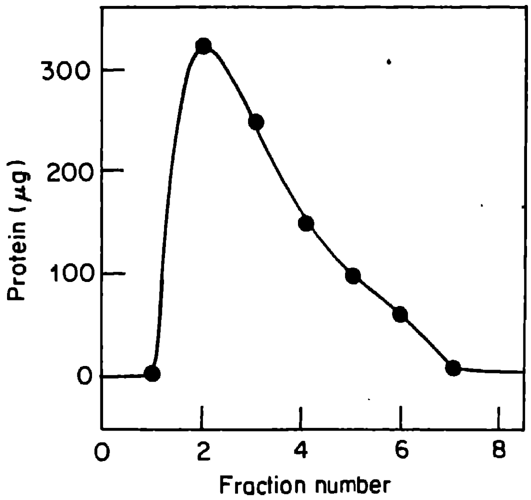

Figure 1. Affinity chromatography for the isolation of cytokinin binding protein from normal rabbit serum.

Removal of bound i^{6}A

The dialysed protein fraction (400 µg) was treated with dextran coated charcoal (0.5% charcoal and 0.05% dextran T. 70 in TBS) for 24 h and then the charcoal was removed by centrifugation (Muniyappa and Adiga, 1980).

Assay for the binding of ^{3}H-i^{6}A by gel filtration

The dextran coated charcoal treated protein fraction was incubated with ^{3}H-i^{6}A chromatographed on Sephadex G-25 and the radioactivity monitored. bovine serum albumin and protein untreated with dextran coated charcoal were also incubated with ^{3}H-i^{6}A and chromatographed separately on the same column as controls. Two peaks were noticed, one at void volume due to protein-bound ^{3}H-i^{6}A and another due to free ^{3}H-i^{6}A region. Table 1 shows the radioactivity i first peak in the experiments. The results show that ^{3}H-i^{6}A bound to the pr fraction only when bound i^{6}A was removed by dextran coated charcoal treatm

Table 1. ^{3}H-i^{6}A binding by chromatography on Sephadex G-25.

Sample	^{3}H-i^{6}A bound* (cpm)
Bovine serum albumin (BSA) (50 μg)	580
BSA (50 μg)+dialysed protein (2 μg)	631
BSA (50 μg)+dialysed and dextran coated charcoal treated protein (2 μg)	4027

* The assay mixture (0.4 ml in TBS) containing ^{3}H-i^{6}A (56,275 cpm) and the sampl incubated at 28°C for 2 h and applied on Sephadex G-25 (1×13 cm) and eluted with After background subtraction, counts in the fraction around void volume were taken i^{6}A bound to protein.

The protein was incubated with ^{3}H-i^{6}A and chromatographed on Sephade 200 (figure 2). Protein-bound ^{3}H-i^{6}A was eluted at the void volume indicati molecular weight above 200,000 for the protein ligand complex.

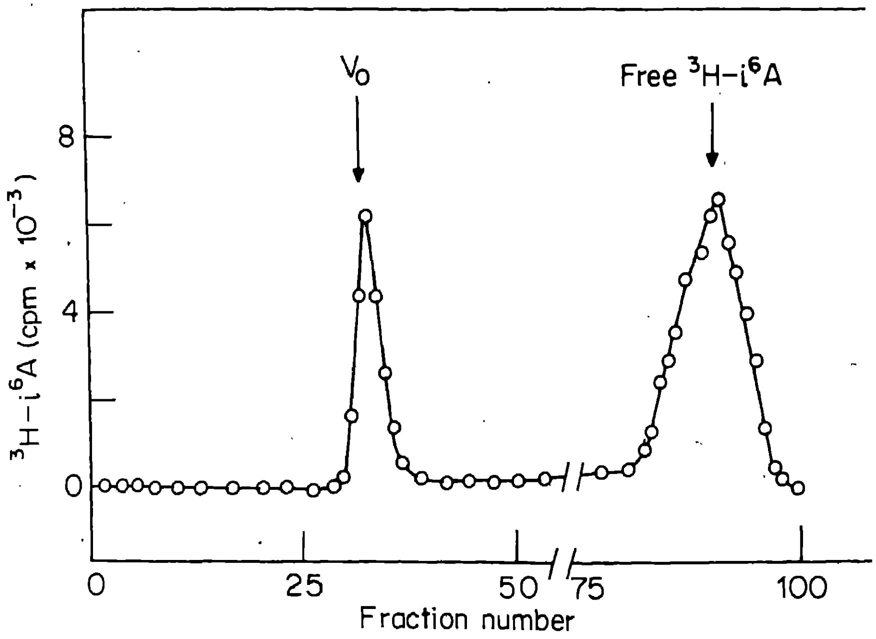

Figure 2. ^{3}H-i^{6}A binding by gel filtration.

The reaction mixture (0.4 ml in TBS) containing ^{3}H-i^{6}A (1125,500 cpm), the protein (6 μ bovine serum albumin (150 μg) was incubated at 28°C for 2 h and applied onto a Sepha 200 column (2×50 cm) pre-equilibrated with TBS and eluted with TBS at a flow rate of 2 Fractions of 2 ml were collected and 0.6 ml aliquots spotted on Whatman No. 3 paper. drying, the papers were counted in a scintillation counter after addition of 5 ml of scintil fluid.

Specificity of the binding of ^{3}H-i^{6}A by nitrocellulose filter assay

The protein fraction was found to bind to ^{3}H-i^{6}A by the nitrocellulose filter assay. The specificity of this binding was determined by measuring its inhibition by non-radioactive cytokinins and related compounds (table 2). The order of inhibition was N^6-(Δ^2-isopentenyl) adenosine >N^6-benzyladenosine >zeatinriboside > N^6-(Δ^2-isopentenyl) adenine> kinetinriboside> adenosine; mevalonic acid and guanosine do not inhibit the binding.

Table 2. Binding of ^{3}H-i^{6}A to the protein fraction in the presence of unlabelled compounds.

Unlablled competitors	Bound ^{3}H-i^{6}A (% of control)
None (control)	100.0
N^6-(Δ^2-isopentenyl) adenosine	12.6
N^6-(Δ^2-isopentenyl) adenine	53.9
N^6-benzyladenosine	32.6
Zeatinriboside	42.1
Kinetinriboside	67.6
Adenosine	68.7
Guanosine	100.0
Mevalonic acid	100.0

The control assay mixture in a total volume of 0.4 ml in TBS consisted of 2 μg of dextran coated charcoal treated protein fraction, 50 μg of bovine serum albumin, 56,275 cpm of ^{3}H-i^{6}A, whereas the experiments contained in addition 2.5×10^{-6}M concentration of the non-radioactive competitor also. Incubation was at 28°C for 2 h and then protein bound radioactivity was determined by nitrocellulose filter assay. Control (no inhibitor) bound had 5536 cpm and 50 μg of bovine serum albumin retained 1862 cpm.

Discussion

About 900 μg of a protein fraction has been isolated from 50 ml of normal rabbit serum by affinity chromatography on AH-Sepharose-pi^{6}Ap> column, using N^6-(Δ^2-isopentenyl) adenosine for elution (figure 1). This protein fraction was present in all the 8 rabbits so far tested. This protein fraction bound tritiated N^6-(Δ^2-isopentenyl) adenosine when the bound non-radioactive N^6-(Δ^2-isopentenyl) adenosine was removed using dextran coated charcoal (table 1). When tritiated N^6(Δ^2-isopentenyl) adenosine and the protein fraction were chromatographed on Sephadex G-200, ^{3}H-i^{6}A bound-protein eluted at the void volume indicating a molecular weight above 200,000 for the protein ligand complex (figure 2). The specificity of the protein fraction when tested by competition experiments in its binding to tritiated N^6-(Δ^2-isopentenyl) adenosine, showed selectivity to cytokinins in general, N^6-(Δ^2-isopentenyl) adenosine being the most preferred one (table 2). Polyacrylamide gel (7%, pH 8.6) electrophoresis of the protein fraction (data not shown) gave two bands, one at the region of the IgG marker and another at the region of the bovine serum albumin marker. ^{3}H-i^{6}A binding activity demonstrated for the protein fraction by gel filtration on Sephadex G-200 is likely to be due to the slow moving band. The conditions used for the removal of the bound ligand from the proteins, might not have succeeded in removing the bound i^{6}A from the protein

corresponding to the faster moving band or they might have denatured the protei
Further studies are needed to clarify these points.

Recently a cytokinin binding protein fraction has been isolated in our laborator
from goat sera (unpublished results of M. Viswanathan and T. M. Jacob).

The discovery that normal mammalian sera contain cytokinin binding protein
is of considerable importance. It gives an indication that N^6-(Δ^2-isopenteny
adenosine may have some normal functions in mammalian systems, probabl
mediated by the binding proteins. It is expected that the cytokinin-bindin
proteins isolated will serve as tools to understand these functions. It is possibl
that cytokinins may be involved in the control of cell proliferation at least in som
mammalian cells for e.g. lymphocytes.

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Changes in native alcohol dehydrogenase activity of *Drosophila* upon treatment with guanidine hydrochloride, urea and heat*

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Abstract. Fifty-two isochromosomal lines of *Drosophila melanogaster* were examined for the existence of additional genetic variations in ADH activity subsequent to treatment with guanidine hydrochloride, urea and heat. A wealth of hidden variation was discovered among and within the Mexican populations of the insect after treatment with the denaturants.

Keywords. *Drosophila melanogaster*; alcohol dehydrogenase; guanidine hydrochloride; urea; heat

Introduction

The existence of clines in the frequency of alcohol dehydrogenase (*Adh*) alleles in *Drosophila* under various environmental conditions offers impressive evidence to support the hypothesis of natural selection affecting enzyme polymorphism. The lines of evidence include reports from Russia (Grossman *et al.*, 1970), United States (Johnson and Burrows, 1976; Johnson and Schaffer, 1973; Vigue and Johnson, 1973; Voelker *et al.*, 1977) and Mexico (Pipkin *et al.*, 1973; 1975; 1976). Pipkin *et al.*, (1976) suggested that the cline of *Adh* alleles depended on selection and gene exchange between Mexican populations of *D. melanogaster* from low and high temperature regions. The above relationships between the gene product and the environmental factors were mainly based on the genetic variation detected by the use of electrophoretic techniques. A variety of techniques revealed additional genetic variation in *Drosophila* (Bernstein *et al.*, 1973; Coyne, 1976; Coyne and Felton, 1977; Gibson, 1970; McDowel and Parkash 1976; Milkman, 1976; Sampsell, 1977; Singh *et al.*, 1976). The number of variants that were observed in a population depended on methodologies used, intensity of effort and the geographical diversity of the strains examined (Koehn and Eanes, 1979).

Recently a statistical analysis revealed cryptic variation in the activity of alcohol dehydrogenase ($\mathrm{ADH^{I}}$ and $\mathrm{ADH^{II}}$) in 12 Adh^{I}/Adh^{I} and 40 Adh^{II}/Adh^{II} isochromosomal lines extracted from 16 Mexican populations of *D. melanogaster* (Miglani,

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* Dedicated to the late Dr. Sarah B. Pipkin of Howard University.

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Abbreviations used: ADH, alcohol dehydrogenase; GuHCl, guanidine hydrochloride; CV, Co-efficient of variation; X, mean; SD, standard deviation; SF, standard error.

1980). The present paper reports the existence of additional genetic variation in ADH activity in the 52 isochromosomal lines subsequent to treatment with various denaturants.

Materials and methods

The sixteen populations of *D. melanogaster* used in construction of isochromosomal lines were: Puebla (Pu), San Louis Potosi (SLP), Tahuacan (Teh), Saltillo (Sa), Actalan (At), Oaxaca (Ox), Cuatla (Cu), Orizaba (Oz), Cordoba (Co), Yanga (Ya), Palenque (Pe), Acayuacan (Ac), Aleman (Al), Coatzocoalcos (Cz), Merida (Me) and Rio Papalopan (Rio). The isochromosomal lines of *D. melanogaster* used in the present investigation were described elsewhere (Miglani, 1980). Flies used for ADH activity were cultured at 25°C. Within 24 h after emergence, forty males were chosen at random and allowed to develop for six days at which time, *D. melanogaster* ADH shows peak activity (Ursprung *et al.*, 1968). The flies were weighed (Sartorium micro-balance) and hand ground in 2 ml of 0.05 M phosphate buffer (pH 7.5). This homogenate was centrifuged at 27,000 *g* for 30 min at 4°C in a Sorvall RC-2B centrifuge. The supernatant, referred to as crude extract was immediately used for ADH activity measurements (Miglani, 1980).

Guanidine hydrochloride (GuHCl) and urea treatment

To a 1 ml cuvette containing 0.89 ml of a 0.7M GuHCl or 1.0 M urea solution (prepared daily in 0.05 M phosphate buffer, pH 7.5) 0.05 ml of the crude extract was added. This mixture was kept in the Gilford recording spectrophotometer (maintained at 30 ±1°C) for 40 s after which 0.05 ml of NAD^+ (27.6 mg NAD^+/ml deionized water) and 0.01 ml of 2-butanol were added. The reaction mixture was vortexed and conversion of NAD^+ to NADH was recorded spectrophotometrically for 2 min. Six replicates were run for each set of homogenates as previously described by Miglani (1980).

Heat treatment

To a 1 ml cuvette containing 0.89 ml of 0.05 M phosphate buffer (pH 7.5) maintained at 45°C, 0.05 ml of crude extract was added. The mixture was vortexed and incubated for 15 min at 45°C. The heat treatment was terminated by adding cold (4°C) 0.05 ml of NAD^+ solution (27.6 mg NAD^+/ml deionized water) and 0.01 ml of 2-butanol. The mixture was again vortexed and ADH activity remaining after treatment was determined as described above.

The native (prior to various treatments) ADH activity for each isochromosomal line was compared with its ADH activity after treatment with denaturants or heat.

Statistical analysis

Mean ($\bar{X}$), standard deviation(s) and coefficient of variation (CV) values for native ADH activity (data reported by Miglani, 1980) and ADH activity after treatment with denaturants (present paper) were calculated only for those populations in which it was possible to extract three or more isochromosomal lines. The differences in the mean and CV values were analysed by standard statistical procedures (Zar, 1974).

In order to separate Adh^{I}/Adh^{I} and Adh^{II}/Adh^{II} isochromosomal lines which differ significantly in terms of the percent ADH activity remaining after treatment with the denaturants, the data were analyzed following the method described by Zar (1974).

Results

A differential decrease in ADH activity was observed after treatment with GuHCl, urea and heat among the Adh^{I}/Adh^{I} (table 1) and the Adh^{II}/Adh^{II} (table 2) strains of *D. melanogaster*. Among all of the 12 Adh^{I}/Adh^{I} lines (table 3), the mean native ADH activity was significantly lowered after treatment with GuHCl, urea and heat. The CV values were significantly higher in all the 12 Adh^{I}/Adh^{I} lines for ADH activity after treatment with GuHCl and urea. However, the CV for the three lines of the Orizaba population significantly increased after heat treatment whereas the 5 lines of the Puebla population did not differ significantly in CV values after treatment.

Table 1. Comparison of ADH activity of isochromosomal lines of phenotype Adh^{I}/Adh^{I} from *D. melanogaster*.

	ADH Activity after treatment with		
Isochromosomal line	GuHCl	urea	heat
Pu-a-1	0.66 (14.1)[a]	0.47 (10.0)[a]	2.07 (44.2)[a]
Pu-b-1	3.90 (87.8)	3.72 (83.8)	1.83 (41.3)
Pu-c-1	1.78 (30.6)	4.46 (76.5)	5.39 (92.4)
Pu-d-1	0.50 (9.6)	0.62 (11.9)	1.83 (35.2)
Pu-d-2	0.78 (14.4)	2.18 (40.2)	3.13 (57.7)
SLP-c-1	2.98 (53.3)	3.80 (68.0)	2.83 (50.7)
Teh-a-5	0.55 (12.7)	1.08 (25.0)	3.95 (91.0)
Cu-a-1	0.47 (10.6)	1.70 (37.8)	4.05 (90.2)
Oz-c-1	0.50 (12.6)	0.75 (18.7)	0.83 (20.7)
Oz-c-5	0.55 (13.6)	0.84 (20.5)	0.48 (11.6)
Oz-d-5	1.28 (32.5)	0.20 (22.7)	2.68 (68.4)
Cz-d-1	1.40 (31.2)	0.20 (4.5)	3.66 (81.4)

The ADH activity is defined as nmol of NADH produced/ml/min/mg of body weight. The crude extract was treated with 0.7 M GuHCl or 1 M urea for 40 s. The heat treatment was for 15 min at 45°C.

[a] the numbers in parentheses refer to percentage ADH activity remaining after treatment with GuHCl or urea or heat. The ADH activity of untreated extracts was taken as 100% (for absolute values refer to Miglani, 1980).

Table 2. Comparison of ADH activity of isochromosomal lines of genotype *Adh*^{II}/*Adh* from *D. melanogaster*.

Isochromosomal line	ADH activity after treatment with GuHCl	 Urea	 Heat
SLP-a-1	0.65 (25.3)[a]	0.99 (38.4)[a]	2.16 (84.0)[a]
SLP-a-2	1.08 (36.0)	2.82 (94.1)	0.72 (24.0)
SLP-a-3	2.14 (82.9)	2.52 (97.8)	0.78 (30.3)
SLP-d-2	1.36 (50.9)	1.38 (51.9)	0.95 (35.4)
SLP-d-4	0.98 (53.6)	0.93 (50.6)	0.69 (38.0)
SLP-d-5	0.68 (27.8)	0.95 (39.3)	1.03 (42.5)
Teh-b-6	1.00 (60.0)	1.45 (87.1)	1.25 (75.2)
Sa-a-1	0.40 (21.7)	0.73 (39.1)	0.57 (30.5)
At-a-1	0.41 (25.4)	0.70 (37.5)	0.50 (31.3)
At-b-2	1.47 (52.6)	2.54 (91.0)	0.57 (20.5)
At-b-3	1.27 (50.6)	2.27 (90.2)	0.43 (17.3)
At-b-4	1.37 (45.7)	2.19 (72.8)	0.65 (21.6)
Ox-a-1	0.48 (31.9)	0.99 (65.6)	0.67 (44.2)
Ox-b-2	0.27 (17.2)	0.58 (36.5)	0.40 (25.4)
Ox-c-4	0.35 (22.5)	0.46 (29.4)	0.93 (59.5)
Ox-d-7	0.30 (17.7)	0.51 (30.2)	1.48 (87.2)
Ox-e-8	0.24 (17.6)	0.51 (38.3)	0.63 (47.2)
Oz-c-4	0.29 (16.0)	0.53 (29.2)	0.66 (36.5)
Co-a-1	0.29 (18.1)	0.74 (46.3)	0.52 (32.3)
Co-b-1	0.28 (15.6)	1.73 (94.4)	0.50 (27.4)
Co-c-1	0.28 (13.1)	0.95 (43.8)	0.51 (23.4)
Co-c-2	0.41 (18.7)	1.08 (48.9)	0.65 (29.3)
Co-c-3	0.35 (13.4)	0.69 (26.6)	2.33 (89.1)
Co-d-4	0.34 (14.6)	0.88 (37.5)	1.01 (43.2)
Ya-a-1	0.50 (34.4)	1.18 (82.0)	0.98 (67.7)
Ya-b-2	0.24 (15.6)	0.42 (27.2)	0.65 (42.3)
Ya-c-1	0.54 (36.6)	0.53 (36.3)	0.59 (40.4)
Ya-d-1	0.71 (51.0)	0.77 (55.0)	0.75 (53.3)
Pe-a-4	0.64 (34.5)	0.82 (47.5)	0.42 (22.5)
Pe-b-4	0.25 (21.6)	0.95 (83.5)	0.27 (24.1)
Ac-a-4	1.77 (82.3)	1.73 (80.4)	1.88 (87.3)
Ac-b-4	1.70 (81.0)	1.62 (77.0)	1.69 (80.4)
Al-a-1	1.17 (47.2)	2.09 (84.2)	1.95 (78.4)
Cz-b-1	0.48 (22.5)	1.39 (64.1)	1.83 (89.7)
Cz-c-1	1.33 (60.2)	1.44 (65.2)	1.58 (71.3)
Me-c-2	0.32 (14.5)	0.57 (26.1)	0.45 (20.7)
Me-d-3	1.40 (49.8)	2.64 (93.6)	2.47 (87.5)
Me-f-5	0.26 (15.1)	0.40 (23.9)	0.29 (17.4)
Rio-b-2	0.48 (73.5)	1.55 (76.8)	1.94 (96.5)
Rio-c-1	1.24 (33.9)	1.24 (67.4)	1.28 (69.3)

[a] Values in parentheses represent per cent activity.

Table 3. Statistical evaluation of the data for the isochromosomal lines of genetype Adh^I/Adh^I

Treatment	X	± S.E.	S.D.	CV	± S.E. (%)
Puebla: 5 lines					
None	5.1	±0.25	0.56	10.9	± 3.47
GuHCl	1.5***	±0.63	1.42	93.1***	±29.48
Urea	2.3***	±0.80	1.79	78.4***	±24.78
Heat	3.3***	±0.20	0.46	14.1	± 4.46
Orizaba: 3 lines					
None	4.0	±0.05	0.09	2.2	± 0.88
GuHCl	0.8**	±0.25	0.43	55.2***	±22.54
Urea	0.8***	±0.04	0.07	8.6***	± 3.53
Heat	1.3*	±0.68	1.18	88.7***	±36.22
12 Lines					
None	4.7	±0.18	0.64	13.6	± 2.78
GuHCl	1.3***	±0.32	1.11	86.3***	±17.62
Urea	1.1***	±0.42	1.47	85.5***	±17.44
Heat	2.7***	±0.40	1.41	51.8***	±10.58

* Significant $p<0.05$
** Significant $p<0.01$
*** Significant $p<0.001$

$\bar{X}$: Mean
SD: Standard deviation
CV: Coefficient of variation

While considering all Adh^{II}/Adh^{II} lines collectively, a significant decrease in ADH activity was observed after treatment with GuHCl, urea and heat (table 4). However, when we considered individual populations, there existed no significant decrease in mean ADH activity after treatment with urea in Actalan and Merida, and after heat treatment in Merida. There was a significant increase in the CV values for ADH activity in all Adh^{II}/Adh^{II} lines (collectively) after treatment with urea and heat with the exception of Actalan population which showed a significant decrease in the CV value (table 4). A significant decrease in the CV value was observed for all Adh^{II}/Adh^{II} lines after treatment with GuHCl.

Analysis of data given in tables 1 and 2 [on the percent ADH activity subsequent to treatment with 0.7 M GuHCl, 1.0 M urea or heat (45°C) for isochromosomal lines raised at 25°C] indicated that the lines having residual activity differing by 60% were statistically different. This established for us, a criterion to designate lines with 80% or greater residual ADH activity as "resistant" and those with 20% or

Table 4. Statistical evaluation of the data for the isochromosomal lines of genotype Adh^{II}/Adh^{II}.

Treatment	$\bar{X}$	± S.E.	S.D	CV	± S.E. (%)
San Louis Potosi					
None	2.5	±0.15	0.38	15.4	± 4.34
GuHCl	1.5***	±0.22	0.55	48.2***	±13.92
Urea	1.6*	±0.35	0.85	53.3***	±15.40
Heat	1.0***	±0.22	0.35	53.0***	±15.30
Actalan					
None	2.5	±0.25	0.61	24.6	± 7.12
GuHCl	1.3**	±0.20	0.49	43.1***	±12.43
Urea	1.9	±0.34	0.83	43.0***	±12.41
Heat	0.5***	±0.04	0.09	8.7***	± 3.08
Oaxaca					
None	1.5	±0.06	0.13	8.5	± 2.68
GuHCl	0.3***	±0.04	0.09	28.9***	± 9.13
Urea	0.6***	±0.09	0.21	35.2***	±11.14
Heat	0.7*	±0.23	0.51	95.5***	±30.19
Cordoba					
None	2.1	±0.15	0.36	16.9	± 4.87
GuHCl	0.3***	±0.02	0.05	15.4	± 4.44
Urea	1.0**	±0.15	0.38	37.4***	±10.79
Heat	0.9**	±0.29	0.72	77.9***	±22.49
Yanga					
None	1.5	±0.03	0.05	3.9	± 1.37
GuHCl	0.5***	±0.09	0.19	39.4***	±13.93
Urea	0.7**	±0.17	0.34	46.6***	±16.48
Heat	0.7**	±0.08	0.17	22.5***	± 7.96
Merida					
None	2.2	±0.32	0.56	25.3	±10.30
GuHCl	0.7**	±0.37	0.65	98.0***	±40.02
Urea	1.2	±0.72	1.24	103.2***	±42.14
Heat	1.1	±0.70	1.22	113.3***	±46.25
40 Lines					
None	2.0	±0.32	0.49	24.5	± 2.70
GuHCl	0.7***	±0.18	0.11	15.3***	± 1.71
Urea	1.2***	±0.11	0.68	56.4***	± 6.31
Heat	1.0***	±0.09	0.61	62.0***	± 6.93

* Significant $p<0.05$
** Signifcant $p<0.01$
*** Significant $p<0.001$

less residual ADH activity as 'susceptible'. All the isochromosomal lines resistant or susceptible to the action of the denaturants used will, here onwards, be referred to as 'cryptic variant' lines. Thirtyfive cryptic variant lines selected according to the above criterion are listed in table 5.

Table 5. Cryptic varient lines resistant/susceptible to the action of GuHCl, urea and heat.

0.7 M GuHCl		1 M urea		Heat (15 min at 45°C)	
Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
Adh^{I}/Adh^{I} Cryptic varient lines					
Pu-b-1	Pu-a-2	Pu-b-1	Pu-a-2	Pu-c-1	Oz-c-1
	Pu-d-1		Pu-d-1	Teh-a-5	Oz-c-5
	Pu-d-2		Oz-c-1	Cu-a-1	
	Teh-a-5		Pz-c-5	Cu-d-1	
	Cu-a-1		Cu-d-1		
Adh^{II}/Adh^{II} Cryptic variant lines					
SLP-a-3	Ox-b-2	SLP-a-2	Co-c-3	SLP-a-1	At-b-2
Ac-a-4	Ox-d-7	SLP-a-3	Ya-b-2	Ox-d-7	At-b-3
Ac-b-4	Oz-c-4	Teh-b-6	Me-c-2	Co-c-3	Me-c-2
	Co-a-1	At-b-2	Me-f-5	Pe-b-5	Me-f-5
	Co-b-1	At-b-3		Ac-a-4	
	Co-c-1	Co-b-1		Ac-b-4	
	Co-c-2	Ya-a-1		Me-d-3	
	Co-c-3	Pe-b-5		Rio-b-2	
	Co-d-4	Al-a-1			
	Ya-b-2	Me-d-3			
	Me-c-2				
	Me-f-5				

Discussion

Ursprung and Carlin (1968), using 6 M GuHCl and 10 M urea, found that the activity of ADH was completely lost as indicated by the absence of isozyme bands after electrophoresis. Therefore, the concentrations used by these investigators would have been inappropriate for the present investigation whose purpose was to detect cryptic variation in ADH activity after treatment with GuHCl,urea and heat. The concentration of GuHCl (0.7 M), urea (1.0 M) and temperature (45°C) used in this study, were, on the average, found to be statistically effective in reducing ADH activity (table 3 and 4).

A positive correlation was found between native ADH activity for Adh^{I}/Adh^{I} lines and ADH activity after treatment with urea (Miglani and Ampy, unpublished). These studies also reported a positive correlation between native ADH activity for Adh^{II}/Adh^{II} lines and ADH activity after treatment with GuHCl, urea and heat.

These relationships suggested that the decrease in ADH activity was possibly dependent on the levels of native ADH activity of the strain. In other words, strains exhibiting a high native ADH activity, on the average, showed a high residual ADH activity after treatment with the denaturants.

Heat stability is affected by a number of genetic factors (Milkman, 1976). Electrophoretically-cryptic allelic differences exist which were expressed as differences in thermostability (Milkman and Pruss, 1975). The present investigation provided additional data showing the presence of cryptic polymorphism for *Drosophila* ADH activity after treatment with GuHCl, urea and heat. The thermal stability of ADH in *D. melanogaster* appeared to be controlled by closely linked genes, if not by the same gene (Sampsell and Milkman, 1977) and on the basis of heat denaturation studies it was further suggested from the magnitude and regularity of differences between free energies of the activation (of the inactivation process) that each thermostability step stemmed from one additional Van der Walls bond (Sampsell and Milkman, 1978).

Some investigators observed that homogenates from Adh^{II} homozygotes were more heat stable than homogenates from homozygotes of Adh^{I} (Day *et al.*, 1974; Gibson, 1970). The present study showed that some Adh^{II}/Adh^{II} isochromosomal lines were more heat stable than some Adh^{I}/Adh^{I} lines and vice versa (table 1 and 2). The degree of thermal stability did not seem to be diagnostic of the electrophoretic class of ADH (i.e. ADH^{I} or ADH^{II}). Anderson *et al.* (1980) made similar observations after heat treatment with *D. melanogaster.* The same pattern was also observed with GuHCl and urea treatments in the present study. Thus some Adh^{I}/Adh^{I} isochromosomal lines were more resistant to the action of GuHCl or urea than some Adh^{II}/Adh^{II} lines and vice versa.

A significant increase in the CV values after treatment with GuHCl, urea and heat was interpreted as evidence to support the discovery of additional genetic variation in isochromosomal lines having approximately the same native ADH activity. These observations clearly indicated that treatment with certain denaturants revealed an enormous amount of cryptic variation at a biochemical level among and within the Mexican strains of *D. melanogaster* examined.

Johnson and Schaffer (1973) suggested that enzyme polymorphisms were often associated with regulatory reactions in metabolism. McDonald and Avise (1976) suggested that at least one of the major functions in the variable levels of enzyme activity might perhaps be of an adaptive significance. Since the cryptic variant lines reported (table 5) differ with respect to a biochemical property of ADH, developmental studies on these thirty-five lines are in progress under variable reaction conditions to examine the regulatory mechanisms involved in genetic modulation of alcohol metabolism and to see if this variation has an adaptive significance.

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Binding of arylsulphatase B to isolated rat liver lysosomal membranes

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Abstract. Binding of arylsulphatase B to isolated rat liver lysosomal membrane has been studied at 37°C. The binding is strongly pH-dependent and is governed by ionic strength of the medium. Experimental evidence is given for the ability of the enzyme to dissociate from the firmly formed membrane-enzyme complex. The dissociation rate is greatly accelerated by raising the buffer molarity. Neuraminidase-treatment of the membrane causes significant reduction in its binding ability to the enzyme. This suggests that sialic acid groups participate, presumably by maintaining surface negativity of the membrane, at a stage of enzyme-membrane interaction process which precedes the internalization of the lysosomal enzymes in the lysosomes.

Keywords. Rat liver; lysosomal membrane; arylsulphatase B; arylsulphatase B-lysosomal membrane complex; dissociation; sialic acid.

Introduction

Receptor-mediated endocytosis is employed to internalise the lysosomal enzymes into the lysosomes from the cells following the formation of secondary lysosomes with high selectivity (von Figura, 1977). This mode of assimilation of enzymes into the lysosomes depends on the phosphomannosyl recognition markers on lysosomal enzymes (Kaplan *et al.*, 1977) and high affinity pinocytic receptors on the cell surface (Sando and Neufeld, 1977), forming a ligand-receptor complex. The binding is highly specific and once bound, the enzyme is rapidly endocytosed and incorporated into lysosomes (von Figura, 1977). Though most of the studies have been limited to intact hepatocytes (Tolleshaug, 1981) or to cultured fibroblasts (Hickman *et al.*, 1974; Ullrich *et al.*, 1978), a direct membrane binding of α-L-induronidase (Rome *et al.*, 1979) and β-glucuronidase (Fischer *et al.*, 1980) to isolated fibroblast membrane receptors have been reported. The binding is reversible and depends mainly upon their immediate environment. Although a plasma membrane receptor for binding of lysosomal hydrolases has been indirectly demonstrated (Kaplan *et al.*, 1977), the presence of such a receptor on lysosomal membrane is not well known.

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In the present investigation, we have described an *in vitro* interaction between partially purified arylsulphatase B and highly purified lysosomal membrane. Association and dissociation of the complex are strongly dependent upon pH and ionic strength. Pre-incubation of the membrane with proteolytic enzymes has no inhibitory effect; however, neuraminidase or sodium periodate partially or completely impair its binding ability.

Materials and methods

p-Nitrocatechol sulphate, trypsin, chymotrypsin and papain were from Sigma, Chemical Co., St. Louis, Missouri, USA. Neuraminidase (*Vibrio cholarae*) was obtained from Grand Island Biological Co., New York, USA. Concanavalin A-Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

Isolation of lysosomal membrane

Normal male albino rats of Wistar strain (body weight, 220-250 g), fed on laboratory stock diet, were used. The animals were sacrificed by decapitation, livers excised quickly, washed and homogenized in 8 vol of 0.25 M sucrose/1.0 mM EDTA. Lysosomes were isolated by the modified differential centrifugation method (Ragab *et al.*, 1967). The final membrane preparation was obtained essentially as described earlier (Pappu *et al.*, 1978) by leaching out the membrane-bound and intralysosomal enzymes by incubating lysosomes with 0.15 M NaCl followed by washing with 0.12 mM and 0.24 mM sodium deoxycholate solutions.

Purification of arylsulphatase B

Rat liver lysosomes were incubated in 0.15 M NaCl for 1 h at 37°C and centrifuged at 27,000 *g* for 10 min. The clear supernatant was concentrated to a small volume using an Amicon PM 10 membrane. Arylsulphatase B was purified by affinity chromatography on a concanavalin A-Sepharose 4B column (1×10 cm) followed by gel filtration on Sephadex G-200 column (2.5×75 cm) as described earlier (Adhikari and Vakil, 1980). In preliminary experiments, it was also ascertained that arylsulphatase A did not bind with the membrane under the experimental conditions employed.

Enzyme assay

Arylsulphatase B activity was estimated using 12 mM p-nitrocatechol sulphate in 0.1 M acetate buffer, pH 5.5, as the substrate (Roy, 1953). One unit of the enzyme activity was expressed as 1 nmol of the substrate hydrolyzed/min.

Protein was estimated as described by Miller (1959).

In vitro binding of arylsulphatase B with lysosomal membrane

In all the experiments, purified membrane preparation (≡1 mg protein) was incubated with arylsulphatase B (≡250 units) for 10 min at 37°C in 10 mM acetate buffer (pH 4.5 or 5.0). The incubation mixture was then chilled, centrifuged (27,000 *g*, 20 min) and the pellet was washed with the same buffer to remove non-specifically adhered enzyme. The pellet was resedimented, suspended in water and the enzyme activity bound to the membrane was determined.

To study the effect of ionic strength on the association of the enzyme-membrane complex, the ionic strength of the acetate buffer (pH 4.5 and 5.0) added to the incubation mixture, was adjusted to molarity varying between 10 and 100 mM.

For dissociation studies, the enzyme-membrane complex was first prepared by incubating the membrane with the enzyme in presence of 10 mM acetate buffer, pH 5.0. The pellet was centrifuged, washed with the same buffer and resedimented. The final pellet was suspended in acetate buffer (pH 4.5 and 5.0) of ionic strength increasing from 10 to 100 mM and incubated for 5 min at 37°C. The mixture was centrifuged, the pellet washed with water and the residual activity bound to the pellet was measured.

Pre-digestion of the membrane with hydrolytic enzymes

For proteolytic digestion, the membrane preparation (about 0.3 mg protein) was incubated with 1 mg papain in 0.1 M tris-HCl buffer, pH 7.6 containing 5 mM cysteine and 1 mM EDTA, or with 1 mg of trypsin or chymotrypsin in 0.01 M tris-HCl buffer (pH 8.1) containing 10 mM $CaCl_2$. Another aliquot of the membrane ($\equiv$0.3 mg protein) was incubated with increasing concentrations of neuraminidase in 0.1 M tris-acetate buffer (pH 6.5) containing 5 mM $CaCl_2$. All the samples were incubated at 37°C for 1 h and centrifuged (27,000 *g*, 20 min), the pellets were washed once with 0.01 M acetate buffer pH 5.0 and used for the binding studies.

For periodate oxidation, the membrane suspensions in distilled water were mixed with an equal volume of 0.02 M sodium-meta-periodate (in 0.1 M tris-HCl buffer, pH 7.4) and the mixture was kept at 4°C. Suitable aliquots were withdrawn at regular time intervals and centrifuged (27,000 *g*, 20 min). The membrane pellet was washed once with water, resedimented and used for the binding studies.

Results and discussion

Purity of lysosomal membrane

Information on the intralysosomal distribution of the lysosomal hydrolases is fragmentary. Although some enzymes are known to be loosely or tightly bound to the membrane (Baccino and Zuretti, 1975), it is not known whether any of them is the structural component of the membrane. Since arylsulphatase B is loosely bound on the lysosomal membrane, most of it can be easily removed by washing the isolated membrane with 0.15 M NaCl (Pappu *et al.*, 1978). Further washing with tris-HCl buffer of high molarity (0.1 M) and alkaline pH (8.0) and subsequent washing with distilled water resulted in the lysosomal membrane preparation, completely free of arylsulphatase B.

Binding of arylsulphatase to lysosomal membranes

In preliminary experiments, the time course and temperature dependence of arylsulphatase B binding to the membrane were carried out (results not shown) The binding was almost instantaneous and the amount of enzyme bound reached a plateau within 10 min. Binding was maximum at 37°C. Fischer *et al.*, (1980) have shown that most of the lysosomal enzymes bind to the membrane with equal efficiency at 37°C.

It was reported earlier (Adhikari and Vakil, 1980) that the binding was pH dependent; the maximum binding took place at pH 5.0. Though at pH values lower than 5.0, binding was not affected, it declined rapidly at higher pH.

Influence of buffer molarity

The ionic nature of arylsulphatase B binding to lysosomal membrane has been demonstrated by studying the effect of increasing buffer molarity, at pH 4.5 and 5.0, on the association and dissociation of the membrane—enzyme complex. At both pH, maximum association was observed at 10 mM and by increasing the molarity of the buffer, a progressive decrease in the binding was observed. However, the inhibitory effect was more at pH 5.0 (figure 1). Similar dependence on ionic strength of the binding of β-glucuronidase has been reported using the model system of a strong cationic exchanger and with tritosomes (Henning *et al.*, 1973).

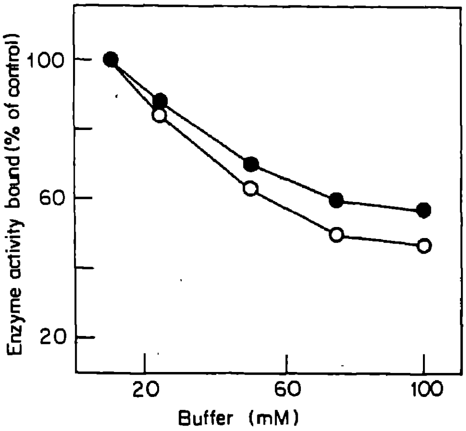

Figure 1. Effect of ionic strength on *in vitro* binding of arylsulphatase B. ph 4.5, (●); pH 5.0, (○).

The enzyme was incubated with the isolated membrane in acetate buffer (pH 4.5 or pH 5.0), with increasing ionic strength. Binding at 10 mM was taken as 100%,

Dissociability of binding is shown in figure 2. The isolated membrane-enzyme complex was suspended in acetate buffers (pH 4.5 and 5.0) of different molarity and incubated for 5 min at 37°C. No dissociation of pre-bound enzyme from the lysosomal membrane was detected at lower (10 mM) buffer molarity and all the enzyme activity was retained in the complex. However, the dissociation of the membrane-bound arylsulphatase B was rapid at higher ionic strength and at 100 mM, about 85% of the bound enzyme was displaced from the membrane within 5 min. Though the pattern of enzyme release as a function of ionic strength of buffer was comparable, it is always higher at pH 5.0. Gonzales-Noriega *et al.* (1980) have demonstrated that enzymes bound to fibroblast monolayers dissociate minimally at neutral pH but maximally at pH 5.0, which is expected in

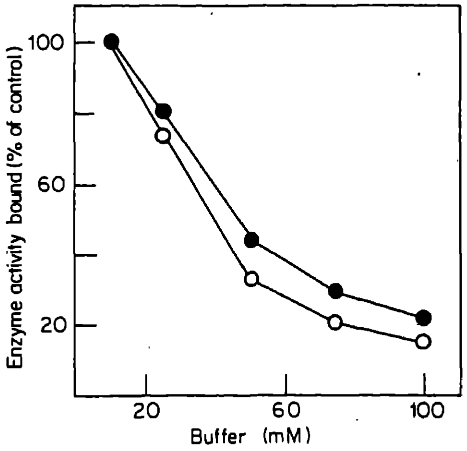

Figure 2. Dissociation of membrane-bound arylsulphatase B at different ionic strengths of the buffer, pH 4.5 (●); 5.0 (○).

After the membrane was incubated (0.01 M acetate buffer pH 5.0) with arylsulphatase B for 10 min at 37°C, the pellet was washed and suspended in acetate buffer (pH 4.5 and 5.0) of varying ionic strengths. The enzyme activity retained on the membrane after incubation at 37°C for 5 min was assayed.

lysosomes. This acidic pH may facilitate the enzyme-receptor dissociation following the delivery of acid hydrolases to lysosomes (Shoji and Poole, 1978). The foregoing results suggest that alterations in the equilibrium of cations and anions affect the enzyme binding to the lysosomal membrane, presumably, by influencing the charges on the cell surface.

Specificity and chemical nature of the binding

Lysosomal membranes contain negatively charged sialic acid groups as well as carboxylic groups of amino acids of the membrane proteins, both of which contribute to the cell surface negativity (Dean and Barrett, 1976). Pretreatment with proteolytic enzymes (table 1) such as trypsin, chymotrypsin or papain had no

Table 1. Effect of proteolytic digestion of the membrane on the binding of arylsulphatase B.

Preincubated with	Enzyme activity bound (%)
None	100.0
Trypsin	92.3
Chymotrypsin	94.7
Papain	92.0

Lysosomal membrane, was pre-treated with the proteolytic enzyme and then used for binding studies.

appreciable effect on the binding ability of the membrane indicating non-involvement of acidic groups from amino acids in the process. However, when the membrane was pre-treated with different concentrations of neuraminidase (figure 3A) and then incubated with the enzyme, the binding ability was decreased with a concomitant release of sialic acid from the membrane. Thus, enzymic removal of sialic acid residues resulted in loss of the binding potency of the membrane. However, Neufeld and Ashwell (1980) have shown that the destructive action of neuraminidase is a repairable lesion in plasma membrane and resialylation can restore its binding ability. The residual binding activity may be attributed either to incomplete enzymic cleavage of sialic acid residues from the membrane glycoproteins or to the fact that perhaps arylsulphatase B binds to the membrane also through some other ligand.

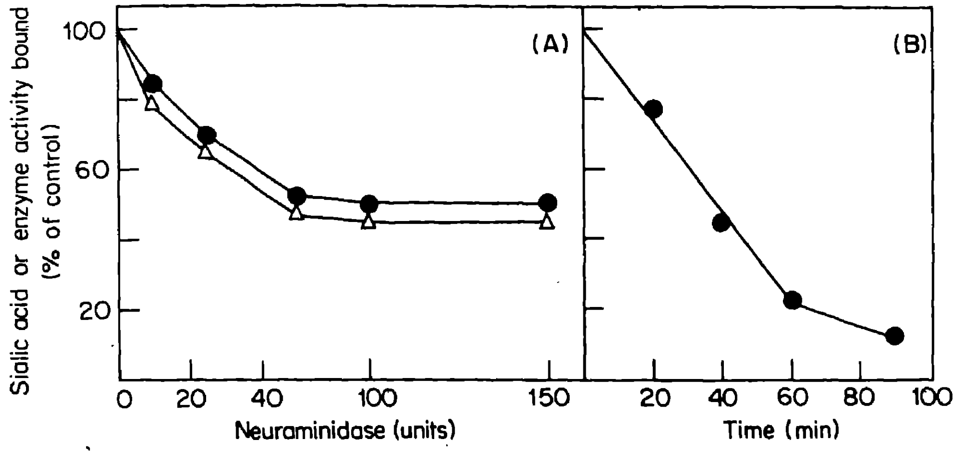

Figure 3. Effect of preincubation of rat liver lysosomal membrane with neuraminidase (A) or with sodium metaperidate (B) on *in vitro* binding of arylsulphatase B. Arylsulphatase B binding, (●) sialic acid, (Δ).

Lysosomal membrane was preincubated with increasing concentrations of neuraminidase at 37°C for 1 h or with 0.01 M (final concentration) Na-metaperiodate at 4°C for different time intervals, washed and used for binding studies. Binding capacity of the untreated membrane was taken as 100%.

In comparison with the neuraminidase treatment, (figure 3B) pretreatment of the membrane with 0.01 M (final concentration) sodium metaperiodate reduces the binding ability of the membrane and the effect is time-dependant. Incubation for 1 h destroyed about 85% of the binding ability of the membrane. Since periodate can oxidise non-specifically all the sugar moieties including sialic acid (Hickman *et al.*, 1974), the possibility of the involvement of other sugar receptor residues on the membrane in enzyme binding processes may not be ruled out. Recently, the presence of such receptors that can recognise carbohydrate residues has also been demonstrated on the lysosomal membrane (Burnside and Schneider, 1980). These may be vitally necessary to ensure the integrity of the lysosomal system during repeated binding events.

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A rapid method for preparation of sarcolemma from frog skeletal muscle

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Abstract. A rapid method for the preparation of sarcolema from frog skeletal muscle has been described. The purified cell segments were transparent and devoid of contractile material. The Na^+, K^+—ATPase and 5'-nucleotidase activities in sarcolemma purified by this method were comparable to those reported for sarcolemmal preparations purified by density gradient centrifugation. The preparation also possessed acid phosphatase, alkaline phosphatase and K^+-activated, ouabain-sensitive p-nitrophenyl phosphatase activities. The cholesterol to phospholipid ratio of the sarcolemma was 0.33, indicating its high purity; further, the preparation was free from mitochondria and contractile proteins.

Keywords. Skeletal muscle; plasma membrane; sarcolemma.

Introduction

Skeletal muscle sarcolemma can be prepared from rat (Kono and Colowick, 1961; McCollester, 1962; Kono *et al.*, 1964; McCollester and Semente, 1964; Rosenthal *et al.*, 1965; Peter, 1970; Kidwai *et al.*, 1973) and frogs (Koketsu *et al.* 1964; Abood *et al.* 1966; Boegman *et al.*, 1970; Narhara *et al*, 1979) by a variety of techniques. Among the more extensively used procedures are the ones which involve prolonged extraction with aqueous salt solutions and drastic homogenization to obtain vesicles of different sizes and separation of the latter by density gradient centrifugation.

Prolonged extraction with aqueous salt solutions results in significant denaturation rendering the preparations unfit for enzymatic studies. When membranes are prepared on the other hand, by drastic homogenization, contamination by intracellular membranes arising out of endoplasmic reticulum or mitochondria is unavoidable. Further more, during homogenization depending upon the composition of the medium, the sarcolemmal vesicles are turned "inside out" or remain "outside out" (Steck *et al*, 1970).

A method is described in this paper which, within 5 h, yields hollow sarcolemmal tube-like structures with good marker-enzyme activities. The method named as "toluene-lithium bromide method" takes advantage, for the first time, of the property of toluene to increase permeability of muscle cells of frog and shorten t time of release of intra-cellular material.

Materials and methods

Adult frogs (*Rana tigrina*) were purchased from local suppliers and kept in a po for a few days before use. All the operations were carried out between 0-5° unless otherwise specified.

Chemicals

Tris, RNA, DNA, bovine serum albumin and enzyme substrates were from Sigr Chemical Co., St. Louis, Missouri, USA, Sucrose (AR) from Polypharma, Bomba lithium bromide from Loba Co., Bombay and toluene from British Drug Hou Ltd., Bombay. All the reagents were of Analytical grade and of the highest puri available.

Light microscopy

The preparations were observed with a phase contrast Olympus microscop (Olympus Optical Co., Ltd., Tokyo, Japan). A 0.1% solution of Janus green w used for staining cell segments during the extraction.

Enzyme assays

5'-Nucleotidase (E.C.3.1.3.5) was assayed using glycine-NaOH buffer (pH 9.0), 1 mM; MgCl$_2$, 5 mM; AMP, 5mM;in a reaction mixture of final volume 1 ml. suitably diluted sarcolemmal preparation was added and incubated for 15 min 37°C. The reaction was stopped by adding 1 ml cold, 10% trichloroacetic acid, t mixture centrifuged and inorganic phosphate determined in the supernatant by t method of Fiske and Subba Row (1925). Na$^+$, K$^+$-ATPase was determined Tris-HCl buffer (pH 7.5), 100 mM; NaATP, 3 mM; MgCl$_2$, 3mM; NaCl, 100m KCl, 10mM and suitably diluted membrane preparation in a total volume of 1 After incubation at 37°C for 30 min, the reaction was stopped by adding 1 ml 10% trichloroacetic acid. The tubes were kept cold and centrifuged to sedime protein and the supernatant was used for phosphate determinations. Ouaba (1mM) was used to study the inhibition of Na$^+$, K$^+$-ATPase.

Phosphomonoesterases (E.C.3.1.3.1) and (E.C.3.4.1.1) at acidic and alkaline p (5.5 and 7.5) were assayed using *p*-nitrophenyl phosphate as substrate as describ earlier (Kidwai *et al.* 1971). Succinic dehydrogenase (E.C. 1.3.99.1) was assay by the ferricyanide method (Rodriguez De Lores *et al.* 1962) and myosin ATPa (E.C.3.6.1.3) was extracted and assayed by the method of Perry (1955).

Preparation of the muscle cell fragments

Adult frogs (*Rana tigrina*) were stunned, decapitated and the muscles from hin limbs were removed and kept in ice-cold 50 mM calcium chloride (pH 7.0); pH the solution was adjusted with 100 mM sodium bicarbonate. Connective tiss and nerves were dissected out. About 50 g of muscle were minced a homogenized for one min in a Sorvall omni-mixer at a speed setting of 240. T homogenate was filtered through a filter described earlier by Kidwai (1978). The residual tissue was rehomogenized, filtered and the muscle cell fragment suspensions pooled and centrifuged at 70 *g* for 5 min in the cold. The sediment was resuspended in ice-cold 0.25 M sucrose (pH 7.0, adjusted with Tris). The absorbance of this suspension was adjusted to a reading of 160 using a red filter in a Systronic Colorimeter. Under phase contrast, the cell segments were dark and of different sizes.

Toluene treatment

Five per cent toluene (v/v) was added to the cell suspension and gently mixed by tilting for exactly 3 min and allowed to settle in the cold for 5 min. The supernatant was decanted and the cell suspension centrifuged at 70 g for 5 min.

Lithium bromide treatment

The sediment was suspended in 150 ml of 0.4 M lithium bromide, pH 8.4, adjusted with 1 M Tris. This suspension was stirred at low speed in the cold for 90 min. The material was centrifuged at 900 *g* for 5 min, the sediment suspended in lithium bromide and stirred for the same period, and once again centrifuged. The resulting sediment was resuspended and stirred for a period of 120 min. The final sediment was suspended in 0.25 M sucrose the pH adjusted to 7.0 by Tris, centrifuged at 900 g for 5 min, the sediment resuspended in 0.25 M sucrose and used for enzymatic and other studies (figure 1).

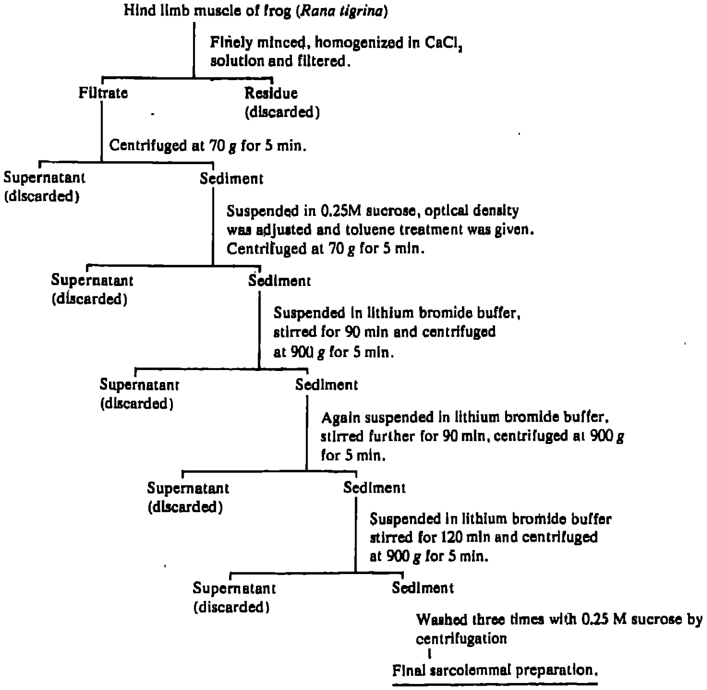

Hind limb muscle of frog (*Rana tigrina*)
Finely minced, homogenized in $CaCl_2$ solution and filtered.
Filtrate
Residue (discarded)
Centrifuged at 70 *g* for 5 min.
Supernatant (discarded)
Sediment
Suspended in 0.25M sucrose, optical density was adjusted and toluene treatment was given. Centrifuged at 70 *g* for 5 min.
Supernatant (discarded)
Sediment
Suspended in lithium bromide buffer, stirred for 90 min and centrifuged at 900 *g* for 5 min.
Supernatant (discarded)
Sediment
Again suspended in lithium bromide buffer, stirred further for 90 min, centrifuged at 900 *g* for 5 min.
Supernatant (discarded)
Sediment
Suspended in lithium bromide buffer stirred for 120 min and centrifuged at 900 *g* for 5 min.
Supernatant (discarded)
Sediment
Washed three times with 0.25 M sucrose by centrifugation
Final sarcolemmal preparation.

Figure 1. Isolation procedure of frog skeletal muscle sarcolemma.

Light microscopy

Cell segments were examined under phase contrast microscope and also by staining with 0.1% Janus green. Figure 2A is a representative phase contrast microscopic picture of a frog muscle cell segment just after homogenization. Cell segments of various sizes were present and all of them were long, cylindrical and broken at the ends. Very little unidentified material was present. Figure 2B is an

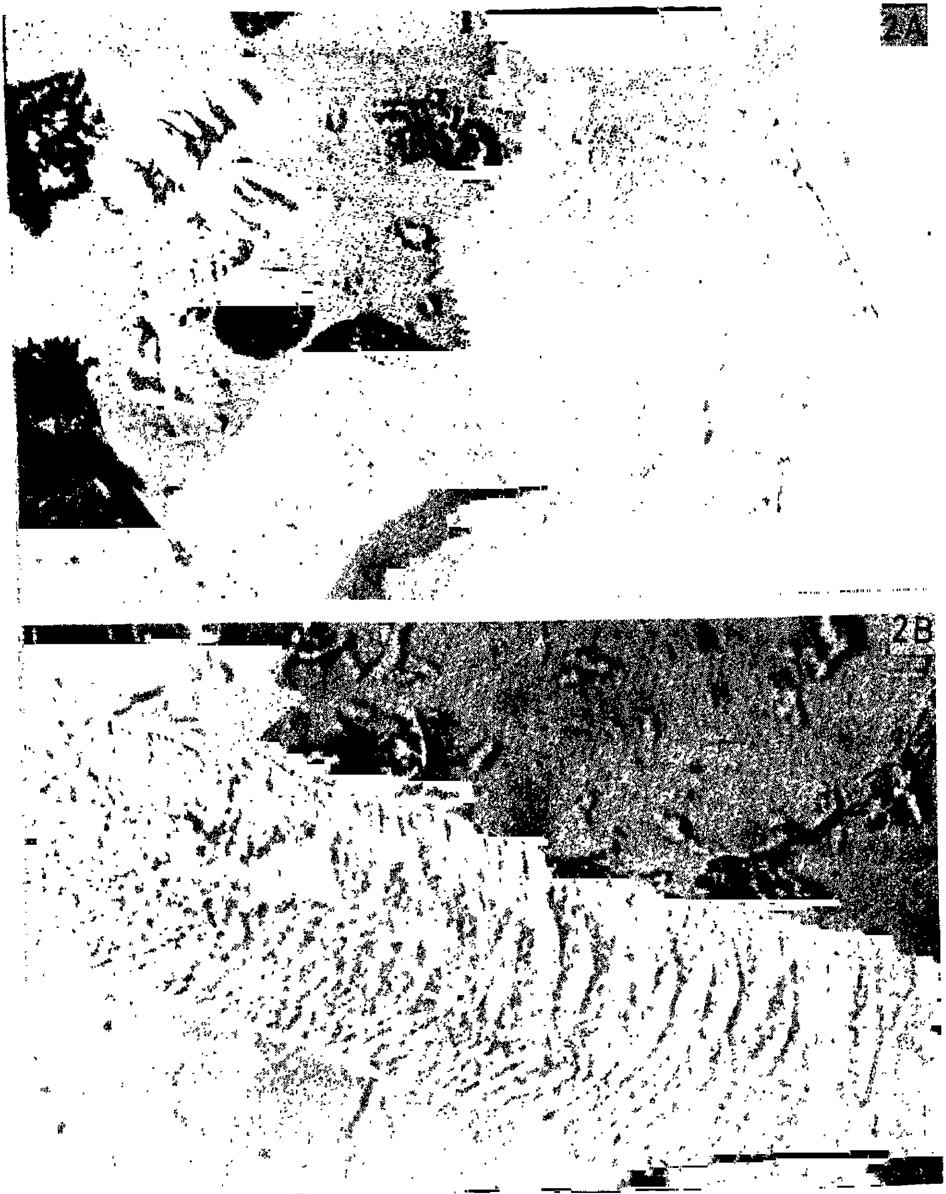


Figure 2. A. Phase contrast photomicrograph of cell segments as seen after homogenization and adjusting the absorbance before toluene treatment (thickness 81 µ). B. A single cell segment after enlargement (thickness 81 µ).

enlarged single cell segment. The cross striations were prominent and the cell segment was dark in appearnce. These cell segments were stained heavily by Janus green. During the first hour of extraction (figure 3A, B), the intracellular

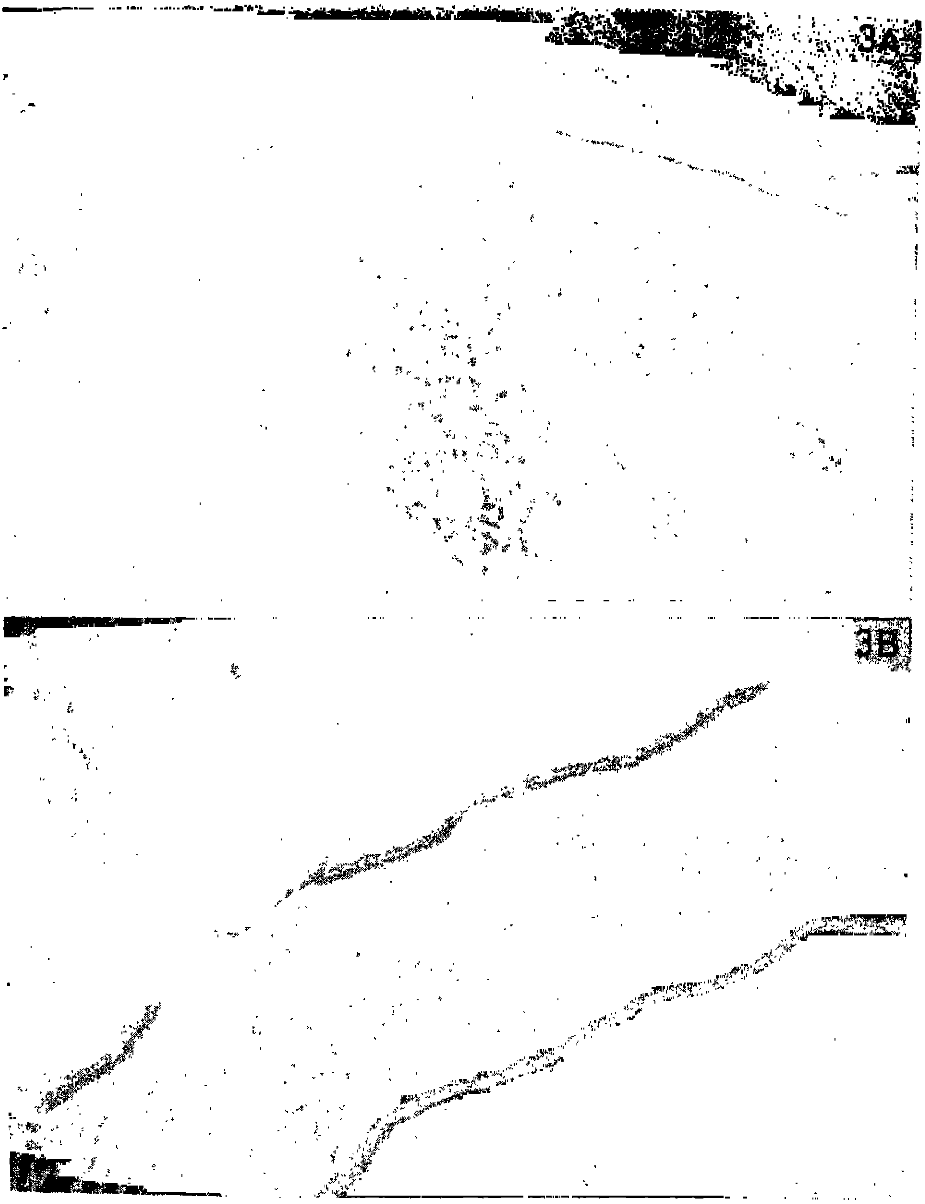


Figure 3. A. Phase contrast photomicrograph of a single cell segment after toluene treatment and immediately after starting extraction with LiBr (thickness 73 μ). B. Protein coming out from the side holes made by toluene (thickness 75 μ).

material started coming out of the holes created by toluene. After 2 h of lithium bromide extraction, the cross striations disappeared and in some cell segments the vicinity of the toluene hole was lighter (figure 4). The intracellular material was seen coming out from the ends of the cell segments as well.

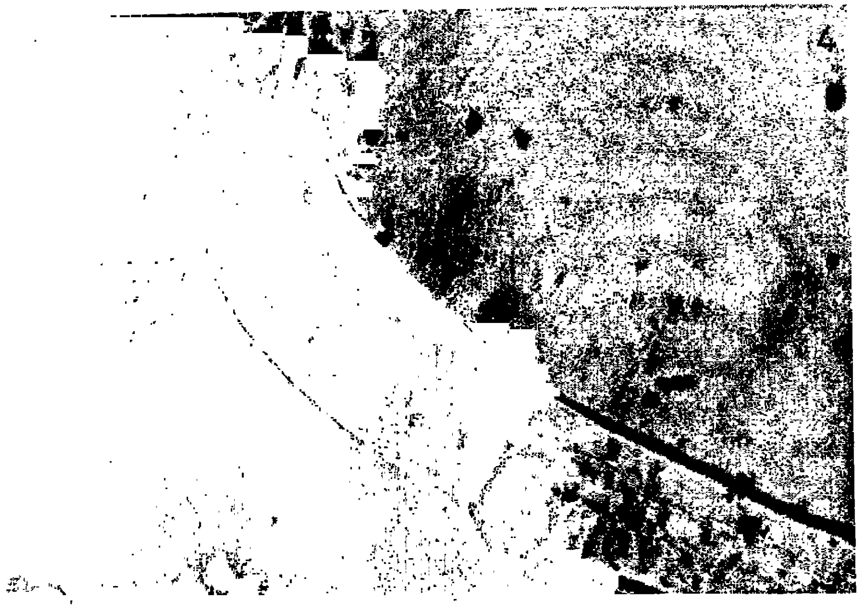
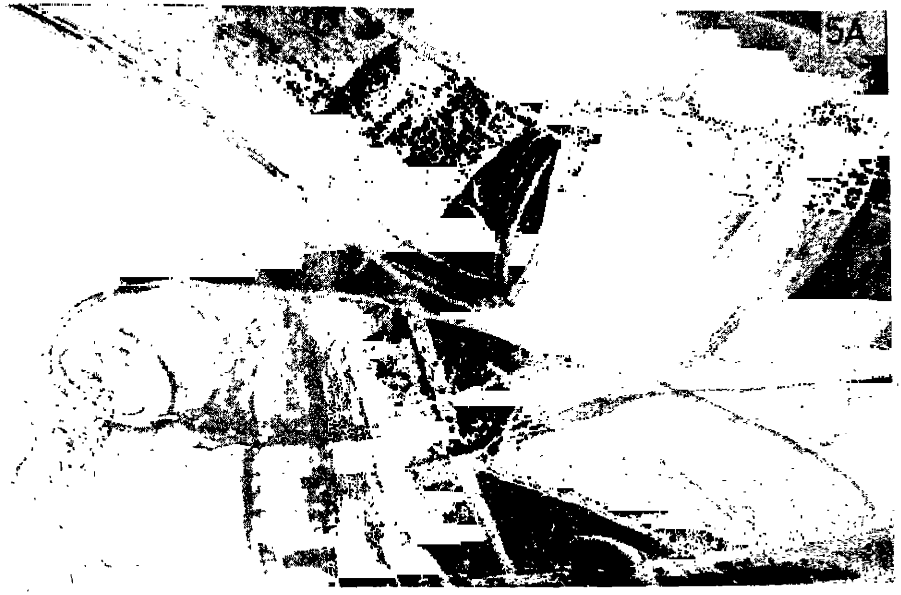


Figure 4. Phase contrast photomicrograph of a single cell segment after 2 h extraction (thickness 54 μ).



At the end of the lithium bromide treatment, the cells were not dark any more (figure 5A, B, C) and gave a cellophane-tube like appearance with one or more holes on the side of these cell segments. The cellophane-tube like structures were not stained by Janus green. The diameter of the sarcolemonal tubes was approximately 80 μ.

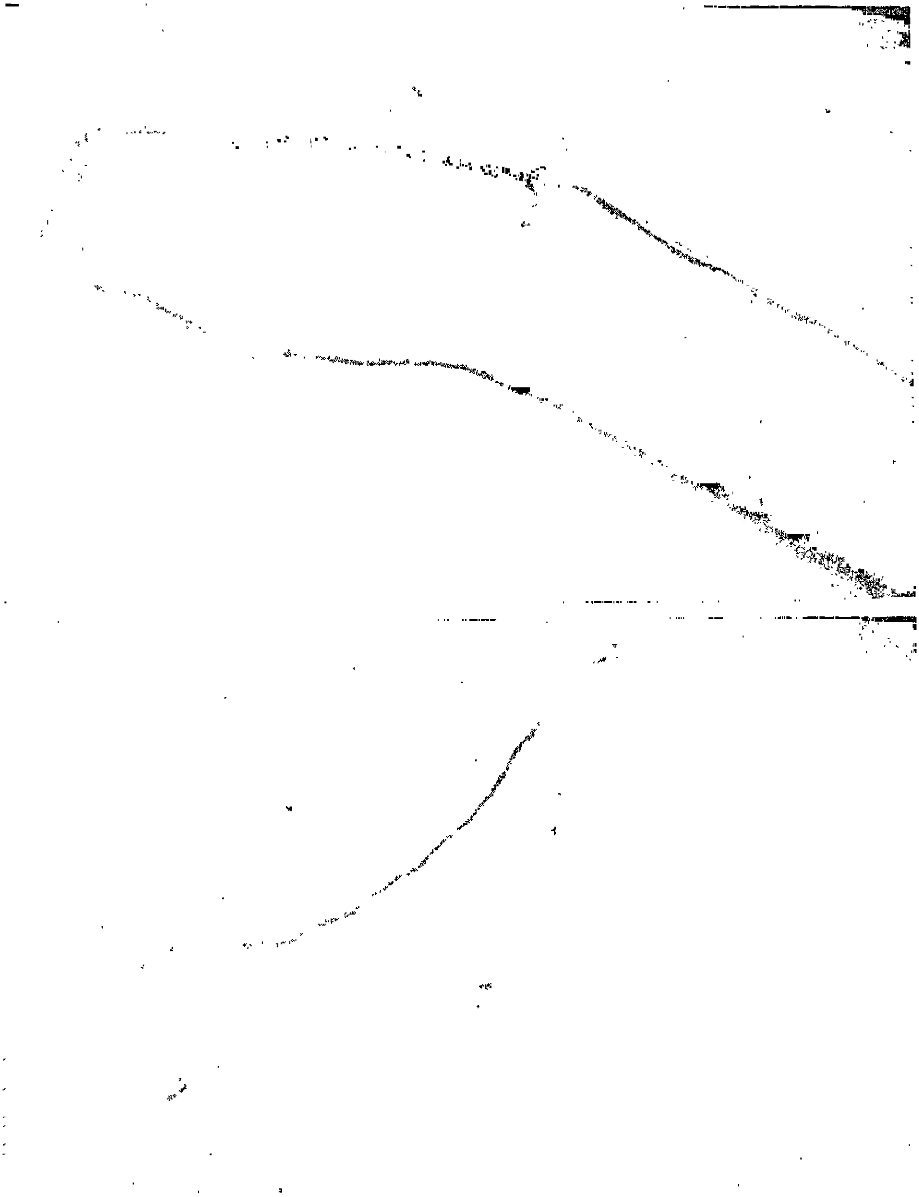


Figure 5. A. Phase contrast photomicrograph of empty cell segments after complete extraction (thickness 76 μ). B-C. Single segment showing holes clearly.

Results

Yield of sarcolemma

The yield of sarcolemma was equivalent to 2 mg protein from 100 mg protein of the homogenate consisting of cell segments. About 0.3 mg protein was recovered from 1 g wet weight of muscle.

Chemical composition

Table 1 indicates the cholesterol, phospholipid RNA and DNA contents of the sarcolemmal preparation.

Table 1. Chemical composition of frog skeletal muscle sarcolemma.

Chemicals	μmol/mg protein	
Cholesterol	0.25±0.092	(4)
Phospholipid	0.85±0.17	(4)
Cholesterol: phospholipid ratio	0.33±0.94	(4)
RNA-phosphorus	0.28±0.09	(4)
DNA-phosphorus	0.12±0.05	(4)

Nucleic acids are expressed as μmol of nucleic acid inorganic phosphate per mg protein. μg lipid phosphate × 25 = μmol phospholipid.

Number in parentheses are the numbers of experiments performed on separate preparations.

Salt-soluble proteins

When purified sarcolemma was treated wih 1 M NaCl for 20 min with constant stirring and centrifuged, no protein could be recovered in the supernatant. However, KCl (100 mM) extracted about 17% of the protein from the preparation.

Deoxycholate treatment

The purified sarcolemma was treated with 0.5% deoxycholate for 20 min with occasional mixing. About 43% of the membrane protein was solubilized and the membrane looked more transparent under light microscopy. Besides holes created by toluene were more clearly visible.

Enzyme markers

In table 2, are given specific activities of Na^+, K^+-ATPase. K^+-activated *p*-nitrophenyl phosphatase (sensitive to ouabain), Mg^{2+}-ATPAse, acid and alkaline phosphatase. Succinic dehydrogenase activity of the preparation was negligible (specific activity 0.02). Myosin ATPase was not detectable suggesting that our preparation was free from mitochondria and contractile proteins.

Table 2. Specific activities of enzymes of frog skeletal muscle sarcolemma.

Enzyme	Specific activities
Mg^{+2}-ATPase	83.52 (6)
Mg^{+2}, Na^{+}, K^{+} [a]	95.06 (6)
Mg^{+2}, Na^{+2}, K^{+}, ouabain (1 mM)	84.66 (6)
5′-Nucleotidase	2.96 (6)
K^{+}-Activated *p*-nitrophenyl phosphatase	0.11 (6)
p-Nitrophenyl phosphatase (alkaline)	2.26 (6)
p-Nitrophenyl phosphatase (acidic)	5.90 (6)
Succinic dehydrogenase	0.02 (6)
Myosin ATPase	0.00 (6)

[a] Specific activities are expressed as µmol phosphorous released/mg protein/h.

Specific activites of ATPase and 5′-nucleotidase are expressed as µ mol phosphorus released per mg protein per h at 37°C. Specific activites of phosphatases are expressed as µmol of *p*-nitrophenyl phosphate hydrolyzed per mg protein per h at 37°C. and the specific activity of succinic dehydrogenase expressed as change in absorbance (due to reduction of ferricyanide) per mg protein per h at 30°C. Number in parenthesis is the number of experiments performed on separate preparations.

Discussion

Earlier methods of isolation of skeletal muscle sarcolemma required extraction for 16-24 h which was followed by drastic treatments. In the present method, the cells were made permeable to charged ions and large molecules by the use of toluene. Toluene is known to make membranes permeable in bacteria, nuclei, hepatocytes and mitochondria (Jackson and DeMoss, 1965; Serrano *et al.* 1973; Hilderman and Deutscher, 1974; Matlib, *et al.* 1979). The muscle cell segments were made permeable to lithium bromide presumably by the removal of sarcolemmal patches from the cell segments. Protein emerged from the ends and also from the holes created by toluene. At the end of the extraction period, the cell segments could not be stained by Janus green. The diameter of the tube-like structure was 100 µ as reported by Koketsu *et al.* (1978).

The preparation retained enzymes known to be present in the plasma membrane from various sources. The activity of Na^{+}, K^{+}-ATPase was very similar to the value reported by Narhara *et al.* (1979) using density gradient centrifugation. The specific activity of 5′-nucleatidase in the present preparation was 3.0 compared with a value of 5.0 reported in rat skeletal muscle (Kidwai *et al*, 1973).

Another criterion for the purity of plasma membrane is a high cholesterol phospholipid ratio. The present preparation had a cholesterol: phospholipid ratio of 0.33. In rat skeletal muscle preparations the values were in the range of 0.11-0.24, (Ashworth and Green 1966; Kidwai *et al*, 1973). The amount of DNA was negligible but a significant amount of RNA was found in agreement with the results as reported by others (Benedetti and Emmelot, 1968). The yield of sarcolemma was much higher than obtained by earlier methods (Kono and Colowick, 1961; Peter, 1970; Kidwai *et al*, 1973). Toluene treatment did not appear to have damaged the surface membrane except to remove patches of membrane and leaving holes.

The method described in this paper has the advantages of yielding sarcolemmal tubes, retaining the shape of the outer membrane with the enzyme activities intact in comparison to earlier preparations (Wattiaux *et al*, 1971).

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The tensile properties and mode of fracture of elastoidin

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Abstract. The tensile properties and mode of fracture of elastoidin, a collagenous protein fibre from the fins of sharks, were compared with those of rat tail tendon fibres, considered to be a pure form of collagen. Elastoidin fibres were stronger than tendon in the dry state whereas the opposite was observed for fibres tested in the wet state. However, elastoidin was stiffer than tendon whether dry or wet. Scanning electron micrographs of the cross-sections and fractured surfaces revealed that elastoidin fibres consisted of fibrils of varying diameter arranged in a lamellar fashion. From the nature of the fractured surfaces, it could be deduced that the primary failure mechanism for elastoidin was probably through a fissuring of the structure.

Keywords. Elastoidin; collagen; tensile properties; fracture surface; scanning electron microscopy.

Introduction

Elastoidin is a fibrous protein present in the fins of sharks. The elastoidin fibres are packed tightly along their length on either side of the cartilaginous radials of the shark fin and they extend to the edge of the fin giving it some stiffness (Alexander, 1974). A study of the fibrillogenesis of elastoidin has shown that it develops in bilateral rows within the dermis (Kemp, 1977). Elastoidin belongs to the collagen class of proteins which are characterised by their high tensile strength in load bearing connective tissues (Wainwright *et al.*, 1976). Collagenous materials exhibit similar high angle x-ray diffraction pattern and appear banded with the characteristic 670 Å periodicity in the transmission electron microscope (TEM). At the optical microscope level, concentric layers of material have been reported in elastoidin when the fibres are viewed in cross-section (Gross and Dumsha, 1958) but the structure has not been discussed in detail. At the molecular level, it has been shown that vast differences occur between elastoidin and collagen from tendon with respect to the x-ray low angle diffraction pattern and the number of interbands within a period as seen in the TEM (Woodhead Galloway *et al.*, 1978). It is found that elastoidin is a simpler system than tendon in that there is no evidence of long range lateral order as in the 32 Å fibril in collagen of tendon and this accounts for the low number of interbands (only three against thirteen for

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Abbreviations used: TEM, transmission electron microscope.

collagen). From chemical analysis, a 75% fraction of collagenous material present in elastoidin (Kimura and Kubota, 1966). In water or 0.9% sali elastoidin exhibits a shrinkage temperature higher than the collagen from rat t tendon. At the macro-level, elastoidin can be considered to be a two-compone system consisting of a collagenous material and non-collagenous protein whi cannot be separated in the solid state but which can be separated into seve protein fractions by chemical treatment (Sastry and Ramachandran, 1965). Ev though the function of elastoidin is mechanical, only its tensile strength has be measured and that too only in the dry state (Mohanaradhakrishnan, 196 Elastoidin can be considered as being responsible for mechanically stiffening t skin from the point of view of molecular organisation alone (Chandross and Be 1979). In the present study, the tensile properties and fracture of elastoidin fibe are compared with those of rat tail tendon fibres, which can be considered to be pure form of collagen, to see how the non-collagenous protein would affect t mechanical properties. It was also felt that such a study would throw more light the structure and function of elastoidin.

Materials and methods

Elastoidin was obtained from fins of the shark, *Carcarius acutus*. The fins, fro adult males, were brought to the laboratory in an ice box. A layer of skin was peel off and elastoidin fibres were taken out with a needle by applying gentle force. T translucent fibres, six to twelve centimetres in length, were cleaned of adheri flesh and rinsed in Ringer's solution containing 0.9% sodium chloride, 0.026 calcium chloride and 0.03% potassium chloride (Mitchell, 1950). The fibres we stored in polythene bags at −20°C. A few drops of toluene were sprayed into t bags to prevent bacterial attack. A similar procedure has been advocated for r tail tendon fibres (Torp *et al.*, 1975) which were also studied in the prese investigation. The storage before testing did not exceed three days.

The mechanical properties of elastoidin and tendon were tested in the 'dry' sta as well after drying them according to the standard procedure (Bergmann an Stein, 1939). Collagen fibres from tendon teased out from adult males an elastoidin fibres were washed in 10% saline for 3 days at 4°C and were subsequent treated with 0.15 M Na_2HPO_4 (which slightly removes non-collagenous componen but not the non-collagenous protein moiety in elastoidin), washed in distilled wat thoroughly and dried in a series of acetone-water mixtures. The shrinkag temperature of the dried samples did not differ significantly from the fresh sampl indicating that the material had not undergone any denaturation during th processing. The dry fibres were conditioned at 65% relative humidity and 24° before testing.

The tensile testing was performed on an Instron tensile testing machine at strain rate of 50% per min on samples of 1 cm length. Since elastoidin fibres ar highly non-uniform only fibres of short lengths with fairly cylindrical appearanc were taken for testing. Data corresponding to fibres exhibiting jaw break wer rejected. The areas of cross section of the fibres were calculated from the densit and weight of the fibres (Ellis, 1969; Torp *et al.*, 1975). To see the effect o disulphide bonds on the tensile properties, elastoidin fibres were reduced at pH 7.

with 1% 2-mercaptoethanol in phosphate buffer for 4 hours (Jiminez *et al.*, 1971) and the fibres were subsequently tested for their mechanical properties after drying. The surfaces of the samples fractured in the dry and wet (in Ringer's solution) conditions were examined in a Cambridge Stereoscan S-150 scanning electron microscope after coating the material with gold in an Edwards E-306 vacuum coating unit with sputtering accessory (Finlay *et al.*, 1971).

Results and discussion

The stress-strain properties of elastoidin in relation to those of rat tail tendon for samples tested in the 'wet' condition are shown in figure 1. Figure 2 shows the stress-strain behaviour of the samples tested in the dry condition. The mechanical properties of elastoidin are distinctly different when compared to those of tail tendon. The low modulus region usually observed for tendon (Viidik, 1973) is absent in the case of elastoidin. This is presumably because elastoidin does not show a crimped structure whereas tail tendon exhibits crimp when relaxed. It may also be noticed that in the wet state elastoidin has less strength and extension

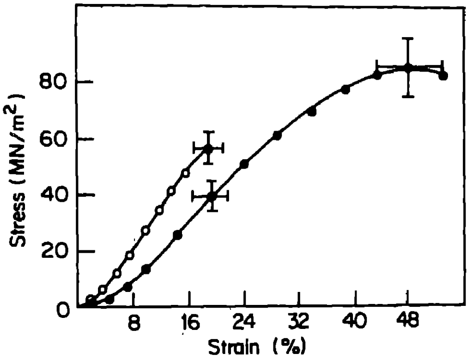

Figure 1. Stress-strain characteristics of elastoidin (O) and rat tail tendon (●) fibres tested wet in Ringer's solution. Standard deviation at intermediate points is approximately the same percentage as at breaking point.

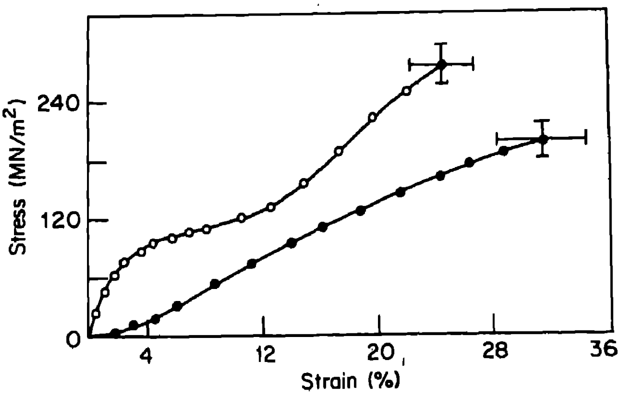

Figure 2. Stress-strain characteristics of elastoidin and rat tail tendon fibres tested dry. Notation same as in figure 1.

at break than tail tendon. The ratio of stress to strain which represents the stiffness or modulus is, however, higher for elastoidin both in the dry as well as in the wet states. In the dry state, the tensile strength of elastoidin is higher that that of tendon. Since the swelling ratio for elastoidin and tendon in water is almost the same, the lower strength of elastoidin here can be attributed only to the interaction of the non-collagenous protein fraction with the collagen fraction in the presence of water.

A curious feature of elastoidin stress-strain behaviour is that in the dry condition, it exhibits a plastic flow region at about 6% strain (figure 2). It has been observed that dry elastoidin fibres exhibit a molecular tilt (Woodhead Galloway *et al.*, 1978). This may be removed by application of strain and the plastic flow region in figure 2 may correspond to the removal of the tilt. It may be mentioned that a similar plastic flow region occurs in the stress-strain characteristics of materials like keratin and Orlon (sometimes called synthetic wool) which have disulphide bonds as in elastoidin. It was however found in the present case that the plastic flow region existed even in the case of elastoidin fibres treated with mercaptoethanol (which selectively removes disulphide bonds).

Elastoidin has a higher percentage of non-collagenous nitrogen showing that the collagen moiety occurs along with other proteins (Kimura and Kubota, 1966). The non-collagenous proteins are strongly bound to the collagen so that a very compact structure is observed. This can account for the higher modulus of elastoidin. It has been concluded from x-ray and TEM studies that there is no lateral order in elastoidin as is observed in tendon *i.e.* the units of elastoidin go on aggregating without limit throughout the fibre whereas there is a 32 Å fibril occurring in tendon (Woodhead Galloway *et al.*, 1978). The intimate binding can account for a compact molecular structure. However, the SEM of cross-sections of elastoidin (figures 3A and B) indicate that there are individual units in elastoidin which range in diameter from 5-30μ. In figure 3 A, it may also be observed that there is a lamellar arrangement of the fibres even though it is not uniform.

The nature of the fractured surface also helps in understanding the properties of the materials. Figure 3C shows the fractured surface of tendon. The appearance is similar for fibres broken in the dry or wet condition. Two possible mechanisms of fracture have been suggested for tendon fracture from TEM studies as pointed out before (Torp *et al.*, 1975). The fibrils which are about 0.45μ in diameter dissociate into subfibrils approximately 0.015μ in diameter and lose their axial register. This is supposed to be the primary failure mechanism. Secondly, fissures may appear in the structure with resultant fracture. The presence of the 10μ filaments (Yannas and Huang, 1972) cannot be established from the picture shown in figure 3C since fibre elements of various diameters are seen ranging in diameter from less than 5μ to 25μ.

Figure 3 (D-F) shows the structure of elastoidin broken in the wet state in Ringer's solution. These may be compared with those of elastoidin broken in the dry state, (figure 3G-I) and with the fracture surface of tendon fibres (figure 3C). The edge of the fracture surface in figure 3D shows a part of the fibre which has broken in shear.

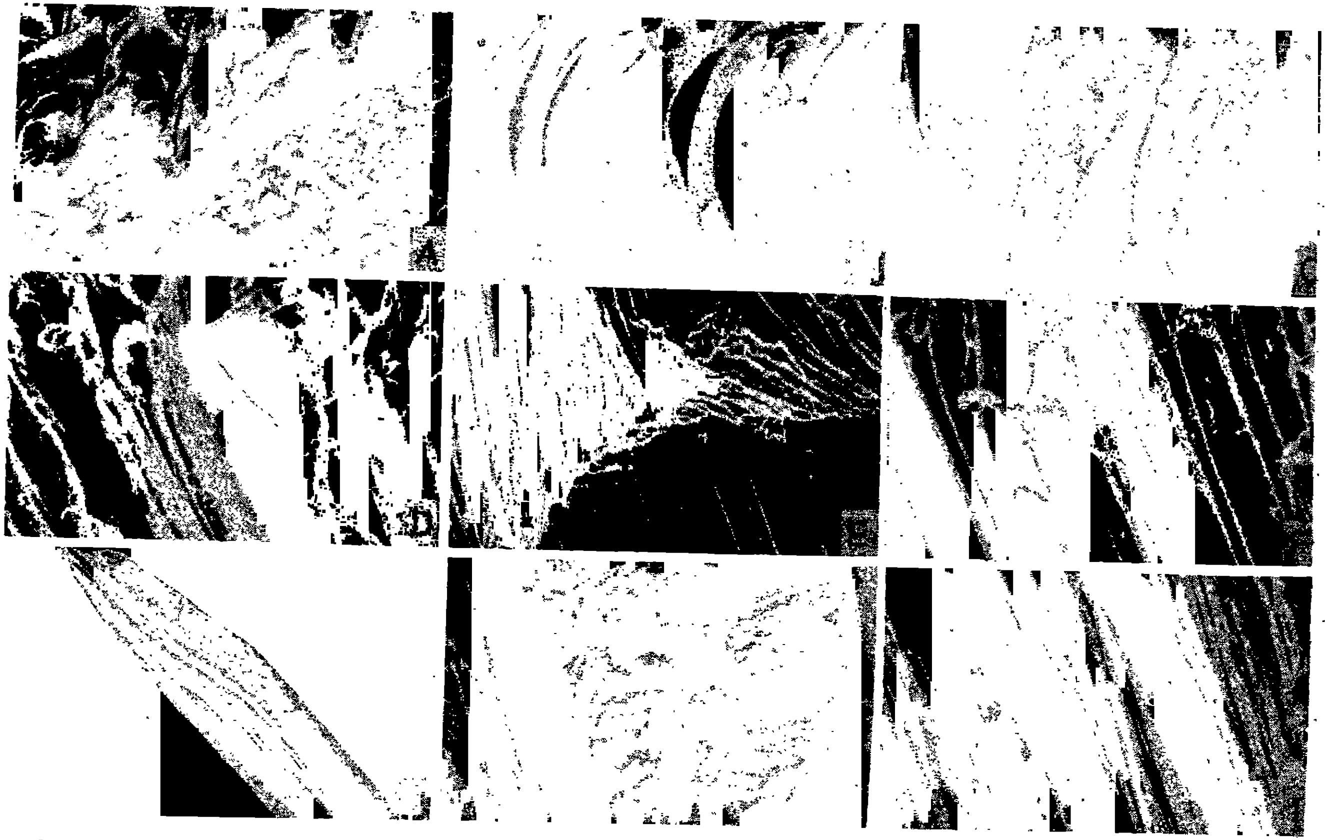

Figure 3. Scanning electron micrographs (SEM) of surfaces of tendon and elastoidin fibres. **A.** SEM of cross section of elastoidin fibre. Arrows indicate fibres of various diameters (X 1000). **B.** SEM of the edge of the cross section shown in 'a' showing fibrillar units (X 100). C. Fractured surface of rat tail tendon showing fibre elements of various diameters (X 100).D-F.SEM of surface of samples tested in the wet state (d, X750; e, X400; f, X540). Arrows in 'e' and 'f' indicate fissures. Lamellae are seen to have sheared and separated at fracture. G-I SEM of surface of samples tested dry. 'g' is a low magnification picture (X 100). In 'h' arrow indicates a fissure. 'h' and 'i' are higher magnifications of regions seen in 'g' (h, X900; i, X 900). Note abrupt break of lamellae in 'h' without shear and helical arrangement in 'i'.

From figures 3E, F it may also be seen that the units have split from the main fibre and fractured. The lamellae also appear separated at the fracture. For the fibres broken in the dry state, there is no shear and the units seem to have broken abruptly (figures 3G, H). A lamellar arrangement of fibre units may be noticed in figure 3H. The lamellar units may be arranged in a helical fashion as seen in figure 3I. This agrees with the observation from x-ray studies of elastoidin fibres (Woodhead Galloway *et al.*, 1978). It is also clear from the figures that the fibrillar units of elastoidin are not cylindrical.

The binding between fibrillar units of elastoidin is affected by the presence of water since shear is more pronounced in the case of samples tested wet. It may also be recalled that the tensile strength is comparatively low in this case (figure 1). The separation of fibre units is not seen to occur in the fibres tested dry(figures 3G, H). From TEM studies, it is found that the elastoidin fibrils break precisely at the periodicities whereas collagen fibrils dissociate into thinner elements at fracture and the periodicities are extended (Mohanaradhakrishnan and Ramanathan, 1966). From these observations, it may be concluded that the fracture mechanisms for elastoidin and tendon are different and it would appear that in elastoidin fissures probably occur and spread so that the sample finally breaks(figures 3E,F. H). This type of fracture is a secondary phenomenon in tendon fracture (Torp *et al.*, 1975) for which the primary failure mechanism is splitting into thinner fibrils. Failure by fissuring of the structure is more likely for elastoidin since there is no definite lateral order at any level.

The higher modulus for elastoidin may have been adapted for the function of the fin. The fins of sharks are held straight by the presence of cartilaginous rays whereas in teleost fishes the fins have rays formed of bone matrix. It is known that the modulus for cartilage is less than that for bone (Wainwright *et al.*, 1976) so that the presence of elastoidin may augment the stiffness of the shark fin. The tensile strength of elastoidin is comparatively low when hydrated but this may not affect the mechanical function of the material in the fin since in the normal physiological state, connective tissues are loaded to less than 20-30% of their breaking stress (Viidik, 1973).

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Model flavoproteins: interaction of riboflavin-5′-mono-phosphate with poly-(α -L-lysine) and poly-(α -L-histidine)*

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Abstract. The interactions of flavin mononucleotide (riboflavin-5′-monophosphate) with two polypeptides, poly-(α-L-lysine) and poly-(α-L-histidine) in water and 0.05 M phosphate buffer were studied by measuring circular dichroism in the pH range 3 to 11. The interation of flavin mononucleotide with the two polypeptides was due to hydrophobic as well as ionic associations and was further influenced by the involvement of the ribityl side chain. The results of the present study have shown that small changes in the environmental conditions of the interacting molecules could modify their mode of interaction considerably.

Keywords. Riboflavin-5′-phosphate; polylysine; polyhistidine; circular dichroism; molecular interaction.

Introduction

Studies on the behaviour of riboflavin-5′-phosphate (flavin mononucleotide, FMN) in flavoproteins and in complexes of FMN with a large number of biological molecules led to the conclusion that the contact of FMN with apoproteins is non-covalent. The types of forces involved in the interactions in such systems (Garcia *et al.*, 1977; Weimer and Niems, 1975) are diverse in character. Since lysine (Bullock, 1967) and tyrosine (Tsibris *et al.*, 1966, Strittmatter, 1961) have been implicated in the interaction between flavin(s) and protein(s) in a flavoprotein it was considered worthwhile to carry out a study of the interaction of FMN with two basic homoplymers, poly-(α-L-lysine) (p-L-Lys) and poly (α-L-histidine) (p-L-His) which might help in understanding some of the features of their interactions in flavoproteins. In the present paper, circular dichroism measurement was used to study the association of FMN with p-L-Lys and p-L-His and the results have been interpreted in terms of specific interactions of the isoalloxazine ring with dipolar functions available on the interacting polypeptides.

Abbreviations used: p-L-Lys, poly-L-lysine; p-L-His, poly-L-histidine; CD, Circular dichroism.

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Materials and methods

Poly-(α-L-lysine) and poly-(α-L-histidine), (average degree of polymerization 1100 and 64, respectively) were from Sigma Chemical Co., St. Louis, Missouri, USA. Riboflavin-5′-phosphate, disodium salt and other chemicals used were of Analytical grade. Stock solution of FMN (1 mg/ml) was prepared in triple distilled water and diluted to 100μg/ml for spectral studies. Solutions of the polypeptides (33.3μg/ml) were used for spectral studies. pH 3.0 and 11.0 were maintained by adding appropriate amounts of hydrochloric acid and sodium hydroxide solutions, respectively. pH 6.8 was maintained using 0.05M phosphate buffer. Circular dichroic spectra in the range 500-180 nm were measured with a Dichrograph Mark III spectropolarimeter. Quartz cells of 0.5 and 0.05 cm path were used. The sensitivity of the instrument was checked before and after the experiment with isoandrosterone (4E 1061, Roussel-Jouan, Paris) solution in dioxane. The base line for each spectrum was drawn separately. All spectral measurements were made at 28°C.

Results and discussion

The electronic absorption spectrum of FMN in water has four bands centered at 220, 264, 370 and 442 nm. The 370 and 442 nm bands have been attributed to $\pi \rightarrow \pi^*$ transition. Intramolecular charge-transfer in FMN leads to the polar excited state and contributes to the 370 nm band (Sun *et al.*, 1972). However, the precise band positions and molar extinction coefficient values largely depend on the environment of the isoalloxazine moiety. On binding with proteins, the 370 and 442 nm bands of FMN shifted to shorter and longer wave-lengths, respectively (Sun *et al.*, 1972). The isoalloxazine ring of FMN, because of its local symmetry, precludes the operation of orbitally degenerate ground or excited states and acquires optical rotatory power only after perturbation in a chiral environment. The circular dichroism (CD) bands corresponding to $\pi \rightarrow \pi^*$ transition of the isoalloxazine chromophore are sensitive to association of all the chiral centres of the ribityl side chain attached to N_{10} and the 370 nm band is mainly affected by the chirality of C_2 (Harders *et al.*, 1974).

Both the polymers, p-L-Lys and p-L-His displayed absorption and CD bands only below 240 nm. For this reason the long wavelength region of FMN absorption, above 240 nm, was considered for interpretation of the present observations. The presence of lysine or histidine (monomers) did not lead to any spectral change in the electronic absorption spectra of FMN. The addition of p-L-Lys or p-L-His also did not influence the position of the 264, 370 and 442 nm bands of FMN under our experimental conditions. However, they were associated with hypo- or hyperchromic effect, depending upon the experimental conditions (table 1).

Circular dichroism spectra of FMN

The CD pattern of FMN at pH 6.8 in the presence and absence of buffer (figure 1) were different from each other. This is interpreted in terms of the ionic environment exerting a strong influence on the conformation of the ribityl side chain. The two CD bands of FMN centered at 262 and 265 nm, appeared only in the absence of the buffer. The CD spectrum of FMN at pH 3.0 (figure 2) was similar in

Table 1. Spectral data of FMN in the presence of p-L-Lys and p-L-His.

Molar Extinction coefficient of FMN	pH 3.0			pH 6.8			pH 6.8 (buffers)			pH 11.0		
	FMN	FMN+ p-L-Lys	FMN+ p-L-His	FMN	FMN+ p-L-Lys	FMN+ p-L-His	FMN	FMN+ p-L-Lys	FMN+ p-L-His	FMN	FMN+ p-L-Lys	FMN+ p-L-His
ε_{442}	11200	9900	12000	11200	10000	—	12000	12400	12800	13200	10400	12200
ε_{370}	9400	9600	9800	9400	8800	—	10800	10800	11200	11400	9200	10400
ε_{264}	29600	31200	30800	30400	27200	—	32800	32400	34400	34800	27800	34800

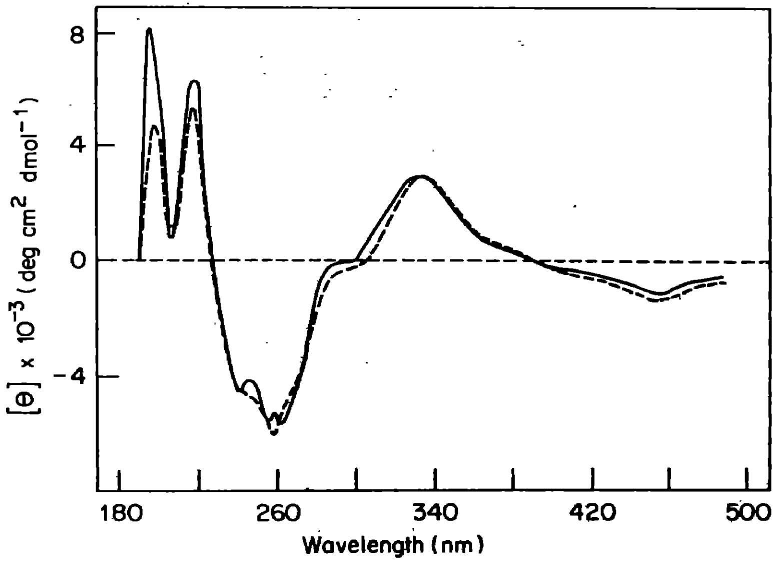

Figure 1. CD spectra of FMN at pH 6.8 ——, in the absence of buffer; - - - -, in the presence of buffer.

shape to that in the presence of buffer at pH 6.8, but the band appeared at 264 nm instead of 258 nm. This may be attributed to the protonation of the N-1 position in the FMN molecule (Tanaka *et al.*, 1967; Dudley *et al.*, 1964). In the deprotonated form at pH 11.0 (Figure 2), the CD spectrum of FMN showed some features of similarity (negative band in the range 240-300 nm) as well as dissimilarity with its CD spectrum at pH 6.8 in the absence of buffer. The strong positive CD band was centered around 332-334 nm at the pH 3.0 and 6.8, both in presence of buffer and water, while, at pH 11.0 it was situated at 338 nm. In each case, this band was followed by a shoulder around 370 nm. The negative long wavelength band appeared at different wavelengths in the 440-456 nm region when the milieu pH varied between 3.0 and 11.0. The CD transitions which appeared in this region may be attributed to the intense 442 nm absorption band (ϵ_{max} 11,200) of unpolarised electronic transition. The appearance of a plateau in the region 290-300 nm at pH 3.0 and 11.0 suggests the presence of a very weak transition. Through fluorescence polarisation and CD measurements the occurance of a 300 nm band has been reported and owing to its low oscillator strength it has been assigned as due to an $n \rightarrow \pi^*$ transition (Miles and Urry, 1968). However, no appreciable shift of this band has been observed in going from polar to non-polar solvents. On the other hand, at pH 6.8, both in buffer and water, FMN exhibited a crossover point (λ_0) at 304 and 300 nm respectively. This transition is more dependent on the configuration of the side chain.

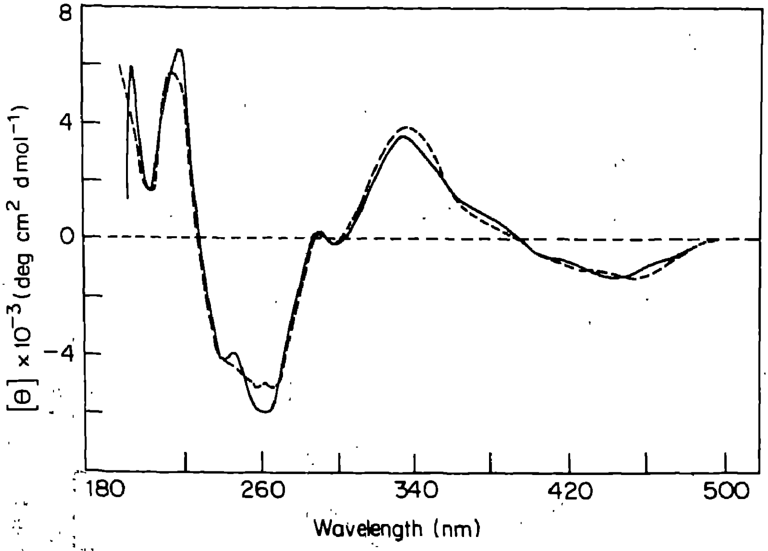

Figure 2. CD spectra of FMN at pH 3.0, ———; and 11.0 – – – –

Circular dichroism spectra of p-L-Lys and p-L-His

The CD spectra of p-L-Lys and p-L-His (figures 3 and 4) under the chosen experimental conditions were found to be in good agreement with the published information (Sarkar and Doty, 1966; Townend *et al.*, 1966; Wolley and Holzworth, 1970; Li and Spector, 1969; Painter and Koening, 1976; Myer, 1969; Burckhard *et al.*., 1976; and Palumbo and Peggion, 1975). p-L-His acquired a random structure at pH 3.0

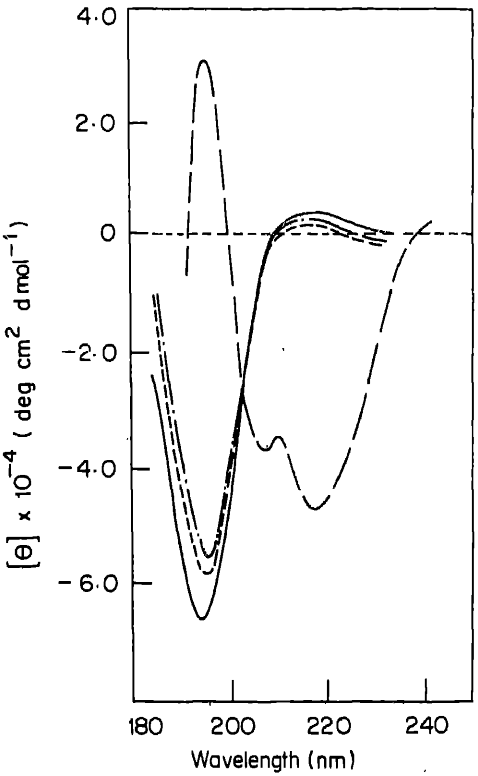

Figure 3. CD spectra of P-L-Lys at pH 3.0, ——— ; 6.8, —·—·— ; 6.8 (buffer) - - - -; and 11.0 — — —.

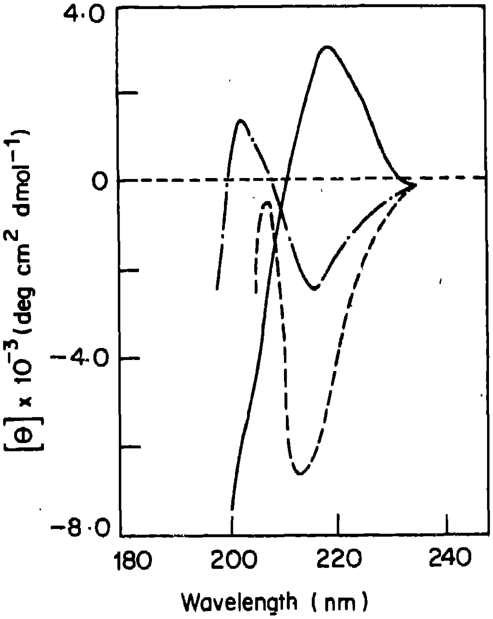

Figure 4. CD spectra of p-L-His at pH 3.0, ———; 6.8 (buffer), —·—·—; and 11.0, — — — —

and 6.8 and a β-sheet structure at pH 11.0. On the other hand p-L-Lys assumed random coil structure at pH 3.0 and 6.8 (both in the presence and absence of buffer) and an α-helical state (*ca* 50%) at pH 11.0.

Circular dichroism spectra of FMN in the presence of p-L-Lys

No spectral change for FMN in the presence of α-L-Lysine has been observed. However, the CD spectrum of FMN in the presence of p-L-Lys is markedly different from that of FMN alone (figure 5). In this interacting system, the two transitions of FMN, the weak one around 300 nm and the strong one at 332 nm were shifted to 304 and 336 nm, respectively. There was an enhancement in the $[\theta]_{304}$ value. The last band of FMN in the long wavelength region was markedly reduced in intensity in the presence of p-L-Lys. These changes can be ascribed to the large changes in the secondary structure of the polymer upon FMN binding.

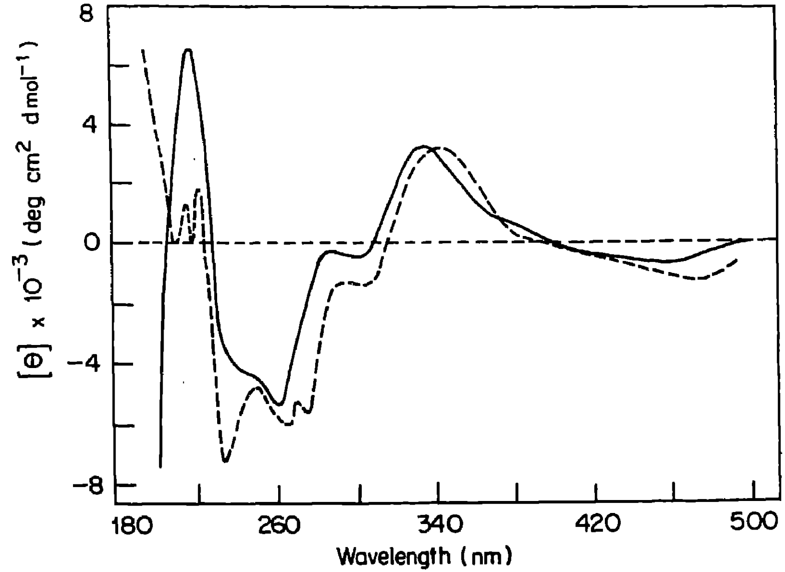

Figure 5. CD spectra of FMN in the presence of p-L-lys at pH 6.8 (in the absence of buffer), ——; and pH 6.8 (in the presence of buffer),-----.

In phosphate buffer (at pH 6.8), the two bands of FMN at 240 nm ([θ]-4500) and 258 nm ([θ]-6000) appeared as three bands at 286 nm ([θ]-7300), 264 nm ([θ]-6100) and 274 nm ([θ]-5700) in the presence of p-L-lys. The λ_0 at 304 nm of FMN at pH 6.8 (buffer) showed a red shift of 12 nm in the presence of p-L-Lys. This was also accompanied by the appearance of a relatively prominent band at 304 nm ([θ]-1400) in the place of a plateau in this region. The two subsequent positive and negative bands at 332 and 456 nm and λ_0 at 370 nm exhibited bathochromic shifts of 14, 20 and 24 nm respectively with accompanying alterations in band intensities in the two bands.

At the two extreme pH values (3.0 and 11.00, the CD patterns of the system, p-L-Lys-FMN (figure 6) were not only different (both quantitatively and qualitatively) from each other but they also varied from the previously described pattern at pH

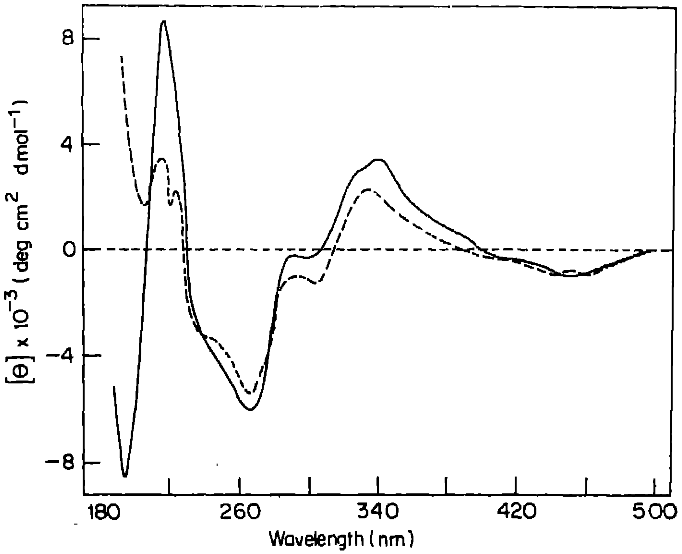

Figure 6. CD spectra of FMN in the presence of p-L-Lys at pH 3.0———; and 11.0- - - -.

6.8. At pH 3.0 the CD band of FMN at 334 nm ($[\theta]+3560$) in the presence of p-L-lys was split into two bands centered at 330 nm ($[\theta]+3000$) and 342 nm ($[\theta]+3300$). Similarly, the plateau around 290-300 nm was changed into a weak band at 300 nm ($[\theta]$-400). Also λ_0 and the last peak at 440 nm shifted to 308 and 450 nm respectively under the influence of p-L-Lys.

In the interacting medium of FMN-p-L-lys, at pH 11.0, the 256 nm band of FMN completely disappeared and the 290-300 nm plateau appeared as a band ($[\theta]_{304}-1300$). The two λ_0's suffered bathochromic and hypochromic shifts of 16 and 6 nm respectively while the 338 nm band appeared at 335 nm with reduced intensity. Instead of the 454 nm band ($[\theta]-1500$) of free FMN, there appeared two bands at 446 nm $[\theta]-1800$) and 460 nm ($[\theta]-1000$) in the presence of p-L-Lys.

Although FMN is similarly bound in several flavoproteins, environmental differences are observed. The addition of p-L-Lys to FMN did not bring about any appreciable change in the absorption behaviour of FMN; however, in the CD spectra, comparatively large changes have been observed. The polymer is only a single polypeptide chain and no bivalent metal ion was incorporated in the system. This ensured that the observed changes in the CD behaviour were only due to the FMN-polymer interaction. These changes are considered to be an indication that a large change in the secondary structure of the polymer has taken place.

The negative CD band located around 300-304 nm in the presence of p-L-Lys, observed in all the four environments is parallel to the situation with chymotrypsinogen (Strickland *et al.*, 1969), which suggests that the FMN binding environment in chymotrypsinogen is probably the same as created by p-L-Lys. In the presence of water at pH 6.8, the 260 nm band has been observed to split into two distinct bands appearing at 264 and 274 nm. The 240 nm shoulder also appeared as a prominent peak, which appeared at 236 nm in the presence of buffer (pH 6.8). This indicated that the free amino groups provided a polar environment for the isoalloxazine ring when it bound to p-L-Lys. In the presence of phosphate buffer, there is hypochromic effect and tailing of the long wavelength band to the red. These effects have also been observed with the protein bound-riboflavin and deoxyriboflavin (Edmondson and Tollin, 1971). Such spectral changes may be interpreted as due to the encapsulation of the FMN molecule in the cavity of the polymer. This has also been suggested by fluorescence studies (Srivastava *et al.*, 1981) which have suggested the presence of multiple sites of flavin—p-L-lys interaction. Secondly, the FMN molecule has been provided a large hydrophilic region at pH 6.8 (in water) and this suggests that the interaction may be occuring between the isoalloxazine ring and the peptide backbone. This is explained by considering the intermolecular stacking or self association of FMN molecules (De Gonzalez and Langerman, 1977) which is further augmented in the presence of p-L-Lys in this milieu or by the interaction between FMN and p-L-Lys; the existing amount of self stacking of FMN molecules remains undisturbed. Spectral changes in the presence of phosphate buffer at pH 6.8 may be interpreted as due to the phosphate ions effectively competing with p-L-Lys molecules. FMN has been known to interact with apoproteins at a faster rate than riboflavin (Edmondson and Tollin, 1971); this has been ascribed to the preferential binding of the phosphate group of FMN to the protein moiety (in flavoprotein) in comparison to the isoalloxazine ring. An analogous situation may be operating in the present case. A comparatively large red shift in the long wavelength region in the interacting systems, suggests that the buffer effect is due to its interference with the self stacking of FMN together with ground state association between FMN-p-L-lys (Srivastava *et al.*, 1981). At pH 11.0, spectral changes observed may be explained in terms of the encapsulation of the isoalloxazine ring in the hydrophobic environment offered by p-L-Lys.

Circular dichroism spectra of FMN in the presence of p-L-His

Monomeric histidine did not show any change in the CD spectrum of FMN. However, substantial changes in the CD spectra of FMN in the presence of p-L-His have been noticed. For this system, shifts in band position, appearance of new bands and disappearance of old ones at the two pH values of 6.8 (buffer) and 11.0 have been observed. Like the FMN-p-L-Lys system, the FMN-p-L-His system also exhibited three CD bands of negative sign between 220-300 nm in the buffer system (figure 7). These bands were centered at 236, 262 and 272 nm. At pH 3.0 and 11.0, the system displayed only two CD bands in this region. The 240 nm band of FMN at pH 11.0 shifted to 236 nm in the presence of p-L-His. At pH 3.0 and 11.0, on the other hand, the presence of p-L-His did not affect the position of the 262 nm band. In phosphate buffer, the 300 nm band of FMN was seen to split into two bands

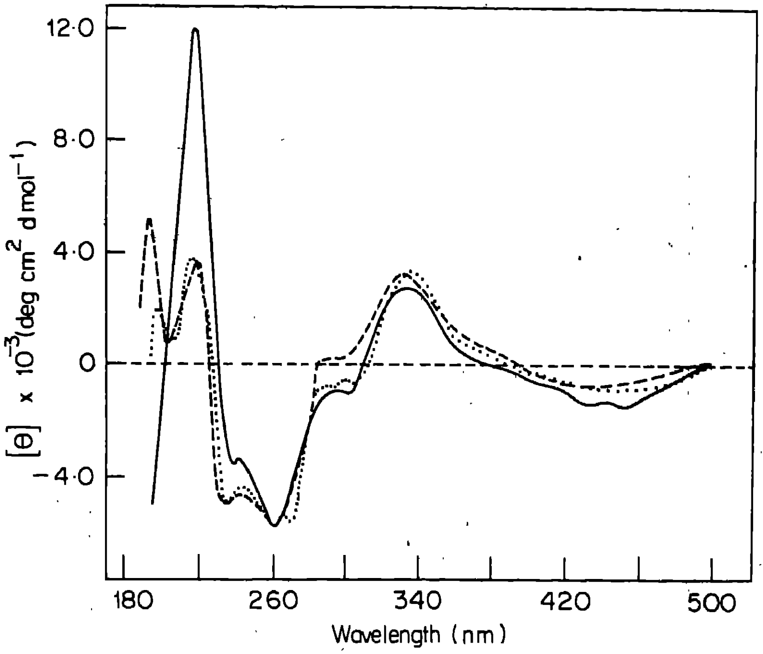

Figure 7. CD spectra of FMN in the presence of p-L-His at pH 3.0, — —; 6.8 (buffer), ...; and 11.0, ——

separated from each other by 12 nm. The above new bands were centered at 292 and 304 nm and were accompanied by an increase in the negative dichroism in each case. The splitting of the 300 nm band could not be observed at pH 11.0 but it showed a two-fold increase in band intensity. At pH 3.0, the sign of the 300 nm band reversed with λ_0 at 284 nm. The shifting of the 332 nm positive band of FMN to 330, 335 and 336 nm was observed at pH 3.0, 11.0 and 6.8 (buffer) respectively.

The CD spectra of the two polymers at pH 3.0 and 6.8 did not show any appreciable difference in their conformation. At the alkaline pH of 11.0 p-L-His exhibited a predominently β-sheet structure and its interaction (in the concentration range 10 to 100 μg) with FMN at this pH was associated with a linear increase in fluorescence (Srivastava *et al.*, 1981), synonymous with a state of nonequilibrium. The sign and shape of Cotton effects associated with lowest ($\pi \rightarrow \pi^*$) transition of FMN at 370 nm in the presence of p-L-His has been affected slightly in a different milieu. The Cotton effect associated with the lowest energy, $\pi \rightarrow \pi^*$ transition is rather more complex. The long wavelength shoulder of FMN appears as distinct band in the presence of p-L-His. These alterations may be considered as resulting from a contribution by two or more vibronic modes to the observed CD envelope. The sign of this fine structure band is dependent on the configuration of the side chain at N-10. The occurrence of fine structure and splitting of the CD band into parts with opposite signs was suggested (Harders *et al.*, 1974) to be due to the presence of different vibronic modes which are in-phase and out-of-phase components of excitons arising from isoalloxazine dimers and aggregates. Formation of FMN

From the foregoing CD spectral observations, it may be concluded that both p-L-Lys and p-L-His interact with FMN in four chosen media in different manners. This property is not shared by their monomers suggesting that such interactions are the overall attributes of their solution conformations and the polymeric nature. The results of this study have shown that a small change in the environmental condition brings about a large difference in the properties of interacting molecules and their nature of interaction.

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ERRATA

Embryonic control of isocitrate lyase activity in cotyledons of germinating soybean

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Volume No. 3, Number 2, p. 145. Please insert the following after the sentence ending 'ponderosa pine (Bilderback. 1974) seedlings.' as a new para.

It is interesting to observe that although the three hormones function by different mechanisms, they cause almost identical increase in enzyme activity and their combined presence produces no additional increase. This would suggest the presence of mechanisms of control which regulate the total amount of the enzyme.

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Fructose diphosphate aldolase-Class I (Schiff base) from *Mycobacterium tuberculosis* $H_{37}Rv$

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Abstract. An aldolase was partially purified from fermenter grown *Mycobacterium tuberculosis* $H_{37}Rv$ cells. The aldolase has a molecular weight of 150,000, possesses a tetrameric structure and cleaves both fructose diphosphate and fructose-1-phosphate, the former being cleaved 17 times faster. The enzyme was inactivated by treatment with $NaBH_4$ in the presence of fructose diphosphate or dihydroxyacetone phosphate suggesting Schiff base formation during its catalytic function. Thiol reagents, EDTA and metal ions had no apparent effect on the aldolase activity. These results show that aldolase is of Class I type. However, this enzyme, unlike the mammalian Class I aldolase, was unaffected by carboxypeptidase A, N-ethylmaleimide and dithionitrobenzoic acid.

Keywords. *Mycobacterium tuberculosis*; fructose biphosphate; aldolase; Schiff base Class I.

Introduction

Class I (Schiff base) type aldolase (EC 4.1.2.13) is generally found in mammals, protozoa and higher plants while Class II type, metalloenzyme, is present in bacteria, fungi and blue green algae (Rutter, 1964; Grazi *et al.*, 1962; Fluri *et al.*, 1967). These two types of aldolases are believed to be of different genetic origin. Recently a few bacteria *viz.*, *Peptococcus aerogenes* (Lephrez and Rutter, 1973), *Lactobacillus casei* (London, 1974), *Mycobacterium smegmatis* (Bai *et al.*, 1975a, b), *Staphylococcus* and *Protococcus* (Goetz *et al.*, 1979) have been shown to contain a Class I type aldolase. However, microbial Class I aldolases differ from mammalian Class I aldolases in several respects such as behaviour towards carboxypeptidase A and thiol reagents. *Esçherichia coli* (Stephen *et al.*, 1978; Stephen and Richard, 1978), *Euglena* (Yeh Chun Mo *et al.*, 1973) and *Chlamydomonas* (Russel and Gibbs, 1967) have both Class I and Class II aldolases. In the present paper we report the purification and properties of a Class I aldolase from *Mycobacterium tuberculosis* $H_{37}Rv$ so as to compare its properties with other Class I type aldolases.

Abbreviations used: FDP, Fructose 1, 6-diphosphate; F-1-P, fructose 1-phosphate; DHAP, dihydroxyacetone phosphate; DTNB, 2,2'-dithiobis 2-nitrobenzoic acid.

Materials and methods

Fructose 1,6-diphosphate (FDP), NADH, α-glycerophosphate dehydrogenase, triosephosphate isomerase, DEAE-cellulose and Sephadex G-150, pyruvate kinase, aldolase, lactic dehydrogenase, bovine serum albumin and malic dehydrogenase were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Acquacide II was procured from Calbiochem, Lucérne, Switzerland.

Growth conditions of the organism

M. tuberculosis $H_{37}Rv$ (NCTC 7416) was grown in Youmans and Karlson (1947) medium in a fermenter. Aeration was at the rate of 5 to 10 ml per min and rotation 300 to 500 rpm. To prevent frothing, 10 to 15 ml silicon oil was added per litre of the medium. The cells were allowed to grow for 14 days after which they were harvested.

Measurement of enzyme activity

The reaction mixture consisted of 2 μmol FDP; 0.3 μmol NADH; 15 μg each of α-glycerophosphate dehydrogenase and triosephosphate isomerase; 50 μmol of glycylglycine buffer pH 7.5 and the enzyme in a final volume of 1 ml.

Aldolase activity was measured by the method of Blostein and Rutter (1963). Correction for NADH oxidase activity was necessary while assaying crude enzyme preparations. The enzyme was incubated with substrate and Mg^{2+} 6 min after which the reaction was stopped by immersion in boiling water for 5 min. Precipitated protein was removed by centrifugation and an aliquot of the supernatant was assayed spectrophotometrically for FDP. Protein was measured by the method of Warburg and Christian (1943).

Activity staining

Polyacrylamide gel electrophoresis was performed in 7% gels by the method of Davis (1964). Samples in 20% sucrose were applied to gels (0.5×6 cm) and subjected to electrophoresis with a constant current of 3 mA per gel. Protein was located using 0.125% Coomassie Blue and activity by the method of Penhoet *et al.* (1969).

Molecular weight determination

The molecular weight of the aldolase was determined by Sephadex G-150 filtration as described by Andrews (1964). The column (30×3 cm) was calibrated using pyruvate kinase (230,000), aldolase (160,000) and lactic dehydrogenase (120,000) from rabbit muscle; bovine serum albumin (69,000) and beef heart malic dehydrogenase (62,000). Two ml of each protein solution (5 mg protein) was passed through the column and the elution volume determined. A solution of 0.25% Blue Dextran (2 ml) was used to measure the void volume.

Dissociation of the enzyme into subunits was achieved by treatment with 1% sodium dodecyl sulphate at 37°C for 3 h (Chan *et al.*, 1967).

Purification procedure

Step 1: Cell-free extract was prepared by sonic disruption of a 20% cell suspension in 0.05 M phosphate buffer, pH 6.0, containing 1 mM dithiothreitol and 1 mM EDTA. Sonication was for 30 min in a Raytheon sonic oscillator at 9 KHz and 4°C. Unbroken cells and cell debris were removed by centrifugation at 20,000 *g* for 30 min.

Step 2: Nucleic acids were precipitated by 2% protamine sulphate (0.1 mg per mg protein), maintaining the pH at 6.0 by the addition of 1 M NH_4OH. The mixture was stirred for 20 min, centrifuged at 20,000 *g* for 20 min and the pellet discarded.

Step 3: DEAE-cellulose chromatography: The protamine sulphate supernatant was applied to a colum of DEAE-cellulose (3×45cm) previously equilibrated with the phosphate buffer containing 1 mM dithiothreitol and 1 mM EDTA. The column was washed with 2 column volumes of the buffer and the enzyme was eluted with the buffer containing a linear gradient of 0.1 to 0.5 M NaCl (total volume, 800 ml) at a flow rate of 30 ml per h. Fractions (3 ml) were collected and monitored for protein and aldolase (figure 1). Fractions with high specific activity were pooled and concentrated using Aquacide II.

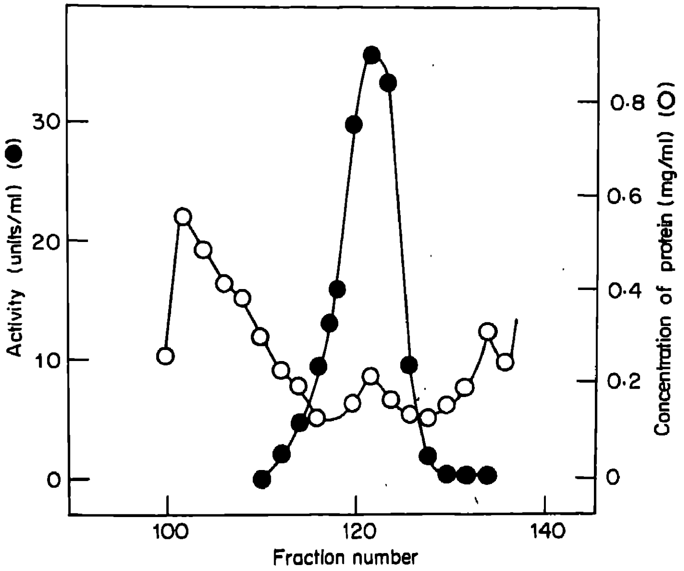

Figure 1. DEAE-cellulose chromatography of *M. tuberculosis* aldolase. Flow rate was 30 ml per h and fraction volme 3 ml. (O) absorbance at 280 nm; (●) aldolase activity.

Step 4: Ammonium sulphate fractionation: The pooled fractions were brought to pH 4.5 by adding 0.2 M acetate buffer, pH 4.0, and treated with solid ammonium sulphate to 80% saturation. The precipitate was collected by centrifugation at 30,000 *g* for 1 h, dissolved in phosphate buffer and desalted using a Sephadex G-25 column.

Step 5: Sephadex G-150 chromatography: The desalted enzyme solution was layered on a Sephadex G-150 column (2.5×30 cm) pre-equilibrated with the phosphate buffer. The column was developed with phosphate buffer (0.05 M; pH, 6.0) containing 1 mM dithiothreitol and 1 mM EDTA at a flow rate of 10 ml per h. 2.5 ml fractions were collected and analysed for aldolase activity and protein content (figure 2). Fractions with maximum aldolase activity were concentrated and stored at −20°C. At this stage, the enzyme was stable for 4 weeks. The enzyme was purified 250-fold with 50% recovery. The results of a typical experiment are summarized in table 1.

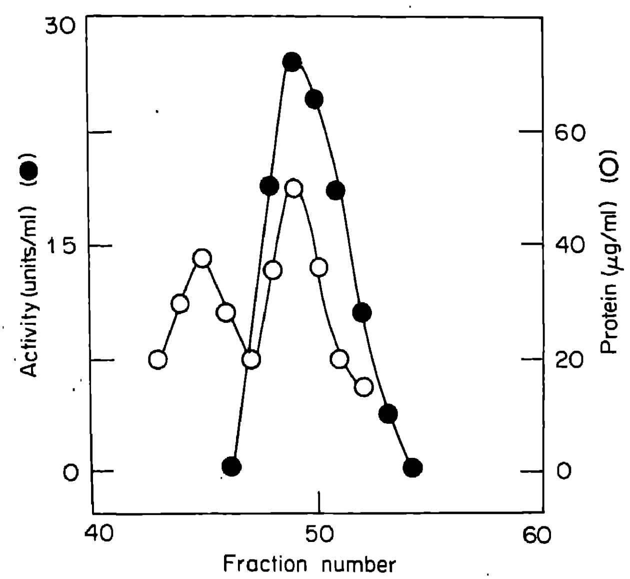

Figure 2. Elution profile of *M. tuberculosis* aldolase on Sephadex G-150 column. Blue dextran was used to measure void volume. Flow rate was 10 ml per h and fraction volume 2.5 ml. (O) absorbance at 280 nm; (●) aldolase activity.

Table 1. Purification of aldolase from *M. tuberculosis* $H_{37}Rv$.

Purification steps	Total protein (mg)	Specific activity[a] (units)	Purification (fold)	Yield (%)
Crude	250.0	2.4	—	100
DEAE-cellulose chromatography	2.5	190.0	75	80
$(NH_4)_2SO_4$ fractionation	1.2	310.0	155	62
Sephadex G-150 chromatography	0.5	601.0	250	50

[a] Specific activity: μmol FDP cleaved/h/mg protein.

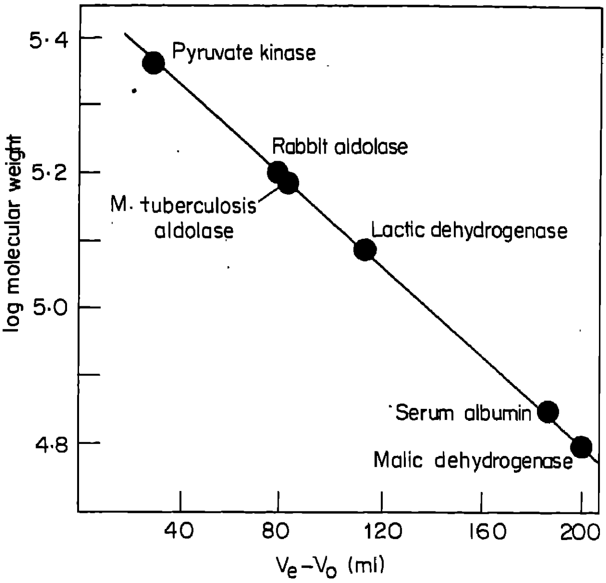

Figure 3. Molecular weight determination of *M. tuberculosis* aldolase by Sephadex G-150 sieving. Ve is the elution volume of the protein and Vo the void volume..

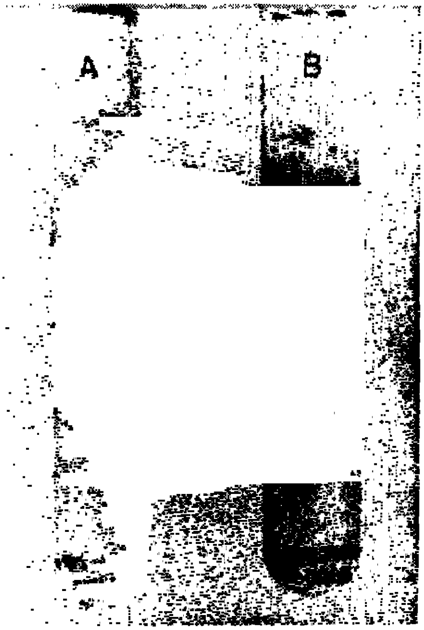

Figure 4. Electrophoretic mobility of subunits of aldolases from *M. tuberculosis* and rabbit muscle. Dissociation of the enzymes into subunits was achieved by treatment with 1% sodium dodecyl sulphate at 37°C for 3 h. A, *M. tuberculosis* aldolase; B, rabbit muscle aldolase.

Results and discussion

Catalytic and molecular properties

The purified enzyme could cleave FDP and F-1-P like the mammalian Class I aldolase. The K_m value for FDP calculated from substrate saturation kinetics was 1.5×10^{-5} µand the V_{max} value was 10.0. An FDP to F-1-P cleavage ratio of 17 obtained for *M. tuberculosis* aldolase was in agreement with that reported for rabbit muscle aldolase (Horecker *et al.*, 1972). Maximum catalytic activity was observed over a pH range of 6 to 9 (table 2).

Table 2. Molecular and catalytic properties of *M. tuberculosis* $H_{37}Rv$ aldolase.

Molecular weight	150,000
Molecular weight of subunit	40,000
Number of subunits	4
Schiff base intermediate	Positive
pH optimum	6 to 9
Km FDP[a]	1.5×10^{-5} M
Km F-1-P	1.0×10^{-2} M
Vmax FDP[b]	10.0
Vmax F-1-P	0.59
FDP/F-1-P	17

[a] K_m values were calculated from double reciprocal plots

[b] V_{max} values are µmol FDP or F-1-P cleaved/min/mg protein.

The molecular weight of aldolase was determined using a Sephadex G-150 column and known protein markers as described under 'Materials and methods'. The elution profile suggested a molecular weight of 150,000 (figure 3).

M. tuberculosis aldolase and rabbit muscle aldolase were treated with sodium dodecyl sulphate and subjected to polyacrylamide gel electrophoresis. Subunits of both the enzymes moved with identical mobility (figure 4). Hence the subunit molecular weight of *M. tuberculosis* aldolase was also 40,000. The molecular weight and subunit molecular weight of the enzyme indicated a tetrameric structure for the protein. As seen in figure 5, the mobilities of both the enzymes were equal on a polyacrylamide gel. The enzyme took an activity stain on this gel.

Effect of $NaBH_4$:

Treatment of the aldolase with $NaBH_4$ in the presence of 8 mM DHAP or 3.68 mM FDP inactivated the enzyme completely (table 3). Incubation of the enzyme with $NaBH_4$ resulted in 20% inhibition only. Loss of activity caused by $NaBH_4$ in the presence of substrate indicated the involvement of lysyl residues in the catalytic process with the formation of Schiff base. Rabbit muscle aldolase, under the same conditions, exhibited a similar property.

Table 3. Effect of $NaBH_4$ on aldolases from rabbit muscle and *M. tuberculosis* $H_{37}Rv$.

Treatment	*M. tuberculosis* aldolase		Rabbit muscle aldolase	
	Residual activity	% activity	Residual activity	% activity
None	10.00	100	520	100
FDP	13.05	130	676	130
$NaBH_4$	8.01	80	480	90
$NaBH_4$+FDP	0.40	4	31	6
$NaBH_4$+DHAP	0.59	6	25	5

Aldolase (1 mg) in 1 ml of Tris-acetate buffer, pH 6.7, containing 3.68 mM FDP or 8 mM DHAP was reduced by the addition of 10 µl of 1 M $NaBH_4$ solution over a period of 20 min and the reaction was stopped by the addition of 5 µl of acetone. Samples were diluted in 0.1 M Tris-acetate buffer (pH 6.7) and analysed for residual enzyme activity.

Effect of carboxypeptidase A

Drechsler *et al.* (1959) have shown that treatment of rabbit muscle aldolase with carboxypeptidase A yielded a product with 7% aldol cleavage activity of the untreated control. However, carboxypeptidase A did not have a deleterious effect on *M. tuberculosis* aldolase (table 4). Microbial Class I aldolases are unaffected by carboxypeptidase A (Lebherz and Rutter, 1973; Stribling and Perham, 1973; Kaklij and Nadkarni, 1974; Bai *et al.*, 1975a, b). Hence the lack of inactivation by carboxypeptidase A appears to be a characteristic of bacterial Class I aldolases pointing out the absence of a—COOH terminal tyrosine residue at the catalytic site.

Table 4. Effect of carboxypeptidase A treatment on aldolases from *M. tuberculosis* $H_{37}Rv$ and rabbit muscle.

Time of incubation (min)	*M. tuberculosis* $H_{37}Rv$		Rabbit muscle	
	Specific activity	Activity (%)	Specific activity	Activity (%)
0	603	100	560	100
20	542	90	22.6	4
40	513	85	10.1	2
60	513	85	10.1	2

Aldolases were incubated with carboxypeptidase A in a molar proportion of 2000:1 at 37°C. Aliquots were withdrawn at specified intervals and activities were measured.

Effect of N-ethylmaleimide

Table 5 represents the effect of incubation of N-ethylmaleimide on aldolases from *M. tuberculosis* and rabbit muscle for a period of 1 h. *M. tuberculosis* aldolase was unaffected while rabbit muscle aldolase activity was remarkably reduced. Two thiol groups of rabbit muscle aldolase reacted rapidly with N-ethylmaleimide and one slowly (Anderson and Perham, 1970). Aldolase of *E. coli* was also inactivated by N-ethylmaleimide (Stribling and Perham, 1973). Studies with N-ethylmaleimide indicated the absence of a direct role for thiol groups in the enzyme catalysis.

Table 5. Effect of N-ethylmaleimide on aldolases from *M. tuberculosis* $H_{37}Rv$ and rabbit muscle.

Time of incubation (min)	*M. tuberculosis*		Rabbit muscle	
	Specific activity	Activity (%)	Specific activity	Activity (%)
0	600	100	560	100
20	648	108	246	44
40	602	100	142	25
60	606	101	58	10

Aldolases were incubated with 5 mM N-ethylmalemide at 37°C, aliquots were withdrawn at specified intervals and activities determined.

The effect of various reagents like sulphydryl reagents and chelating agents on *M. tuberculosis* aldolase were studied (table 6). Dithiothreitol, o-phenanthroline, N-ethylmaleimide, 8-hydroxyquinoline, glutathione, iodoacetic acid, *p*-chloromercuribenzoate, DTNB, β-mercaptoethanol and EDTA were without any effect on the enzyme activity. Inhibition by 1 mM EDTA is a characteristic property of Class II aldolases of bacteria and fungi. However, *M. tuberculosis* aldolase was unaffected by 1 mM EDTA. Higher and lower concentrations of EDTA were tested and were also found to have no effect on the enzyme. These results showed that the aldolase is of Class I type. Further, 0.1 mM K^+ did not elicit any enhancement of the aldolase activity. The divalent ions Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} were also without any apparent effect on the enzyme.

Properties like inactivation by $NaBH_4$ in the presence of substrate, lack of inhibition by EDTA, a broad pH optimum, cleavage capacity for both FDP and F-1-P and lack of activation by K^+, together with its molecular weight and subunit structure prove that the aldolase of fermenter-grown cells of *M. tuberculosis* is of Class I type. The enzyme differs from other Class I aldolases in that it is unaffected by carboxypeptidase A, N-ethylmaleimide and DTNB. The same differences have been reported for other microbial Class I aldolases also. Even though Schiff

Table 6. Effect of sulphydryl reagents, chelating reagents and metal ions on *M. tuberculosis* aldolase.

Additive	Concentration (mM)	Activity (%)
None	—	100
Dithiothreitol	1.0	114
o-Phenanthroline	0.25	75
N-Ethylmaleimide	2.5	100
8-Hydroxy quinoline	1.0	102
Glutathione	1.0	110
Iodoacetic acid	0.5	94
p-Chloromercuribenzoate	0.5	80
β-Mercaptoethanol	1.0	100
Dithionitrobenzoic acid	2.0	97
EDTA	1.0	98
K^+	0.1	102
Zn^{2+}	0.1	96
Mg^{2+}	0.1	104
Mn^{2+}	0.1	34
Co^{2+}	0.1	45
Ni^{2+}	0.1	64
Cu^{2+}	0.1	74
Fe^{2+}	0.1	84

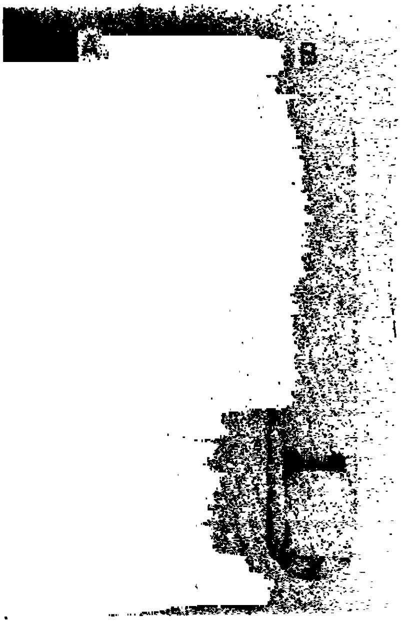

Figure 5. Activity staining of aldolases on polyacrylamide disc gel electrophoresis. Twenty micrograms of the aldolases were applied to 7% gels (0.5×6 cms). A, *M. tuberculosis* aldolase; B, rabbit muscle aldolase.

base formation is a common property shared by all Class I aldolases, the active site of bacterial aldolases may be different from those of mammalian aldolase Detailed investigations of the amino acid residues at the catalytic sites alone ca bring to light the mechanism of action of bacterial Class I aldolases.

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Studies on carbohydrate moieties of *Aspergillus niger* glucoamylase II: Isolation, purification and characterization of glycopeptides

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Abstract. Six glycopeptide fractions namely GP-C_1, GP-C_2, GP-C_{3a}, GP-C_{3b}, GP-D_1 and GP-D_2 were isolated after exhaustive digestion of glucoamylase II (Glucozyme) from *Aspergillus niger* with pronase. They were purified using gel-filtration, high-voltage paper electrophoresis and ion-exchange chromatography on Dowex-50 and Dowex-1. They appeared homogeneous on electrophoresis under different conditions of pHs. The molecular weights ranged from 1600 and 4000 for these glycopeptides. All of them contained serine at the N-terminal end. Serine and threonine were the major amino acids with glycine, alanine, proline and tryosine present as minor constituents. Carbohydrate analysis revealed the presence of different sugars. Based on this, the glycopeptides were grouped into three types: (1) GP-C_1 and GP-C_2 containing mannose, glucose and galactose; (2) GP-C_{3a} and GP-C_{3b}, containing mannose, glucose and glucosamine; and (3) GP-D_1 and GP-D_2 containing mannose, glucose, galactose and xylose. Most sugar constituents in each glycopeptide occured in non-integral ratios implying a microheterogeneity of the carbohydrate moiety in *Aspergillus niger* glucoamylase II.

Keywords. Glucoamylase; *Aspergillus niger*; glycopeptides.

Introduction

Glucoamylase (α-1, 4-glucan glucohydrolase EC. 3.2.1.3) from fungal sources is a microbial glycoprotein that has received considerable attention particularly because it is used in the commercial production of dextrose. It has been isolated from fungal sources and studied by several investigators. In most instances two forms have been reported (glucoamylase I and glucoamylase II). The gross carbohydrate composition of these glycoproteins from *Aspergillus* species has been investigated (Pazur, *et al.*, 1971; Lineback *et al.*, 1972; Venkataramu *et al.*, 1975; Manjunath and Raghavendra Rao, 1979). Lineback *et al.*, (1972) have

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Abbreviations used: FDNB, 1-Fluoro-2, 4-dinitrobenzene; DNP-amino acids, dinitrophenyl amino acids; TNBS, trinitrobenzene sulphonic acid; Gly, glycine; Val, valine; Arg, arginine.

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provided some information on the structure of the carbohydrate moieties. However, further studies such as the isolation of glycopeptides, the sequence and distribution of the carbohydrate moieties were not conducted. Only one such study seems to have been made on the glucoamylase of *Rhizopus javanicus* (Watanabe and Fukimbara, 1974a, 1974b, 1975; Watanabe, 1976).

In the present investigation, an attempt has been made to prepare glycopeptides from *A. niger* glucoamylase II in order to obtain information on the size, number, composition and structure of the carbohydrate units and their distribution in the enzyme molecules, and the nature of the bonds linking them to polypeptide.

Materials and methods

Dowex-1×2 (200-400 mesh), Dowex-50 ×2 (200-400 mesh) and 1-fluoro-2,4-dinitrobenzene (FDNB) were purchased from the Sigma Chemical Company, St. Louis, Missouri, USA. p-Dimethylaminobenzaldehyde (recrystallised by us according to Boas, 1953) and dinitrophenyl amino acids (DNP-amino acids) were from the Council of Scientific and Industrial Research Centre for Biochemicals, V.P. Chest Institute, Delhi. Pronase (1,170,800 tyrosine units/g) was from Kaken Chemicals Co. Ltd., Tokyo, Japan. Biogels were obtained from Bio Rad, Granada Hills, USA. Trinitrobenzene sulphonic acid (TNBS) was purchased from Eastman Chemicals, Rochester, New York, USA. Glucose, galactose, maltose, pyridine (distilled over KOH and ninhydrin before use), phenol (distilled over Zn turnings before use) and benzidine were from the British Drug House, Bombay. All other reagents and chemicals used were of analytical grade unless otherwise described.

'Glucozyme', a crude preparation of glucoamylase prepared from *A. niger* was obtained from Anil Starch Products, Ahmedabad. Glucoamylase II from this source was purified to homogeneity as described earlier (Manjunath and Raghavendra Rao, 1979).

Total carbohydrate by phenol-H_2SO_4 reaction, neutral sugars by gas liquid chromotography, amino sugars by the Elson Morgan reaction were determined as described earlier (Manjunath and Raghavendra Rao, 1979).

For amino acid analysis, glycopeptides were hydrolysed for 24 h at 110°C with constant boiling HCl (5.8 N) in evacuated tubes. Amino acids were determined in a Beckman Model 120 B amino acid analyser (Kedenburg, 1971). Free amino acids were determined using trinitrobenzenesulphonic acid as described by McKelvey and Lee (1969). The N-terminal amino acid was determined by the procedure of Sanger (Schroeder, 1967).

Paper electrophoresis of peptides was carried out on Whatman No. 3 MM paper (27×60cm) at various pH (Ambler, 1963): (a) pH 1.8, acetic acid-formic acid-water, 10:3:107 (v/v) (b) pH 3.4, pyridine-acetic acid-water 1:8:9 (v/v) and (c) pH 6.4, pyridine-acetic acid-water, 225:7.5:2018 (v/v). Electrophoresis was usually carried out for a period of 1 h at 3000 V, 90-110 mA in a Savant high-voltage electrophoresis apparatus. Peptides and amino acids were detected by staining with ninhydrin-cadmium acetate reagent (Heilmann, *et al.*, 1957). Carbohydrate was detected using the periodate-benzidine reagent (Gorden, *et al.*, 1956).

Molecular weights of glycopeptides were determined by Biogel filtration (Spiro, *et al.*, 1976). Biogel P-10 equilibrated with 0.1M pyridine-acetate buffer pH 5.0 was packed into a column (0.9×126cm). The glycopeptides (0.1 mg) were dissolved in 200 µl of 0.1 M pyridine-acetate buffer and applied to the column. They were eluted with the same buffer and the fractions containing carbohydrates were detected by the phenol-H_2SO_4 reaction. Blue Dextran was used to determine the void volume. Glucose, raffinose, Gly-Val-Arg, insulin, glycopeptide (unit A and B) prepared from the Pronase digest of thyroglobulin and glycopeptide prepared from the pronase digest of ovalbumin were used as standards.

Molecular weights of some glycopeptides were also determined by analytical ultracentrifugation (Klainer and Kegeles, 1955). For centrifugation analysis, the glycopeptide (1%) was dissolved in 0.01M potassium phosphate buffer (pH 7.0) containing 0.09 M KCl and the Archibald runs were made at 21740 rpm (Jamieson, 1965).

Results

Preparation of glycopeptides from glucoamylase II

Treatment with pronase: Initially attempts were made to prepare glycopeptides from native glucoamylase II. The native enzyme was treated with pronase (ratio of pronase: glucoamylase II, 1:50) (pH 7.5-8.0) for 20 h and the digest was then passed through a calibrated Sephadex G-200 column. The first fraction eluted had a molecular weight of 65,000 (while the native enzyme had a molecular weight of 80,000), contained most of the carbohydrate and had more than 60% the original glucoamylase, 1:200). The reaction mixture was incubated for 36 h at 37°C in the amino acids but was not digested any further and was too large for structural studies.

Therefore glucoamylase II was denatured before proteolysis (Arai *et al.*, 1966) with the following modification. A solution of 1.4 g of GA II in 56ml water was brought to pH 1.9 by carefully adding 6 N HCl at 25°C. The mixture was then kept at 60°C for 1 h, cooled to room temperature and the pH rapidly adjusted to 8.0 with 2 N NaOH. Calcium acetate was added to the cloudy suspension to a concentration of 0.01 M and proteolysis was started by adding 7.0 mg of pronase (pronase: glucoamylase, 1:200). The reaction mixture was incubated for 36 h at 37°C in the presence of toluene to prevent microbial growth. The pH of the solution was maintained at 8.0 by the addition of 2 N NaOH. The progress of the digestion was followed by TNBS analysis of the increase in amino groups and it levelled off after 33 to 36 h. The digest was then concentrated by lyophilization.

Biogel filtration: The lyophilized pronase digest was subjected to gel filtration on a column of Biogel P-30 as described in figure 1a. The recovery of the applied sample was 87% with respect to neutral sugars. The major carbohydrate peak (fractions 28-55, pooled and lyophilized), designated GP-A, contained 85% carbohydrate.

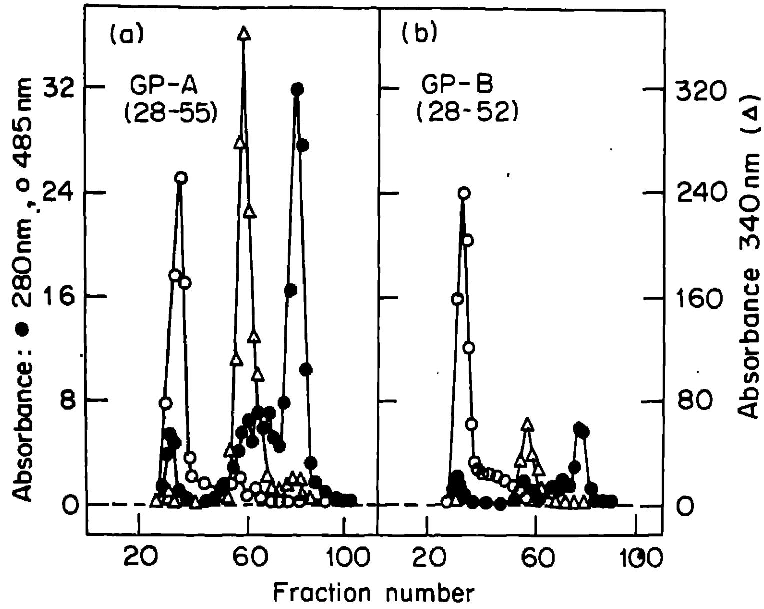

Figure 1. Gel filtration patterns of first (a) and second pronase (b) digest of glucoamylase II.

A column of Biogel P-30 (2×120 cm) equilibrated with 0.01 N acetic acid was used. Fractions of 5ml were collected: Carbohydrate (O) was monitored by the phenol-H_2SO_4 reaction; free amino groups (Δ) detected by TNBS method. Absorbance (●) at 280nm.

Pronase P used in the present investigation was free of carbohydrases and contained approximately 2.5% carbohydrate (phenol-H_2SO_4 reaction). During gel filtration on P-30, pronase elutes with the carbohydrate-rich fraction (GP-A). However, the amount of pronase used was only 1% of glucoamylase II protein. This contributed only a negligible amount (approximately 0.35 mg) of carbohydrate compared to the glucoamylase carbohydrate (250 mg).

Second pronase treatment and Biogel filtration: Fraction GP-A was digested a second time with pronase as described above. The digest was chromatographed again on a Biogel P-30 column. The elution profile is shown in figure 1b. The carbohydrate containing fractions (designated GP-B) were pooled and concentrated by lyophilization. The recovery of neutral sugars was almost quantitative.

Preparative paper electrophoresis: GP-B was further purified by preparative paper electrophoresis by streaking 1 mg of material (as glucose)/cm width on Whatman No. 3 MM paper and electrophoresing at pH 1.8 (43 volts/cm for 60 min). The glycopeptide zones were located on test strips with periodate-benzidine and ninhydrin reagents. Two distinct glycopeptide bands were observed: GP-C, 10cm and GP-D, 4.5cm from the cathode. The areas of the electropherogram showing a positive reaction to both the ninhydrin and periodate reagents were cut out separately, eluted with water and freeze-dried. Several other ninhydrin-positive bands on the same electropherogram gave negative tests with the periodate reagent. The total recovery of glycopeptides was about 88% based on the carbohydrate content.

Chromatography on Dowex-50X2: The glycopeptide GP-C was chromatographed as shown in figure 2a. Three carbohydrate peaks were observed in the effluent. The distribution of carbohydrate as percentage of the total recovered neutral sugar is as follows: peak 1 (GP-C$_1$), 33%; peak 2 (GP-C$_2$), 20%; and peak 3 (GP-C$_3$), 38%. The fractions constituting these peaks were separately pooled, concentrated and further purified on a column of Dowex-1X2.

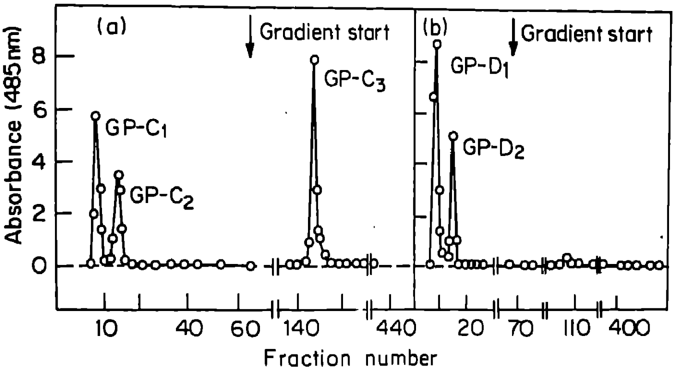

Figure 2. Dowex-50X2 chromatographic patterns of glycopeptides GP-C(a) and GP-D(b).

The cation exchange resin Dowex-50WX2 was prepared as described by Schroeder (1967). The resin was equilibrated in 2mM pyridine-acetate buffer pH 3.1 and packed into a 0.9X110 cm column. The sample of glycopeptide was applied with the above buffer and the column was washed with same buffer. Around fraction 70, a linear gradient of 500ml each of 2mM pyridine-acetate pH 3.1 (in the mixing chamber) and 700mM pyridine-acetate pH 5.8 (in the reservoir) was started, resulting in both salt and pH gradients. Fractions of 3ml were collected at a flow rate of 45ml/h. Glycopeptides were detected by the phenol-sulphuric acid reaction.

GP-D was also similarly chromatographed. The chromatographic pattern obtained is shown in figure 2b. This material which was homogeneous by electrophoresis, gave rise to two carbohydrate-positive peaks. The distribution of carbohydrate as percentage of the total recovered neutral sugar is: peak 1 (GP-D$_1$), 80% and peak 2 (GP-D$_2$), 20%. The fractions were separately pooled and concentrated, and further purified on a column of Dowex-1X2.

Dowex-1X2 chromatography: The glycopeptides GP-C$_1$, GP-C$_2$, GP-C$_3$, GP-D and GP-D$_2$ were chromatographed separately on Dowex-1X2. The elution pattern was similar to that described by Hilschman and Craig (1965) and is shown in figure 3. Glycopeptides GP-C$_1$, GP-C$_2$, GP-D$_1$ and GP-D$_2$ all eluted as one major and several minor peaks. The major fractions were pooled separately and freeze-dried. The minor glycopeptide fractions amounted to about 3% of the total glycopeptides and were not therefore pursued any further. Glycopeptide GP-C$_3$ was eluted in two carbohydrate-containing fractions (Figure 3c). The distribution of

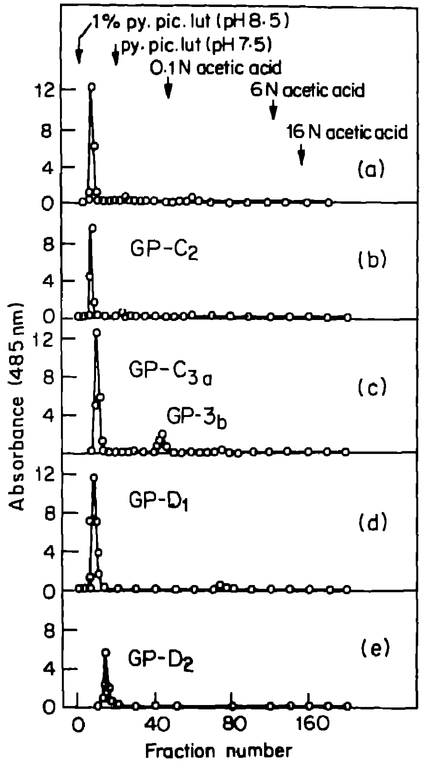

Figure 3. Dowex-1×2 chromatographic separation of glycopeptides. a, GP-C; b, GP-C_2; c, GP-C_3; d, GP-D, and e, GP-D_2.

The anion exchange resin Dowex-1×2 was prepared as described by Schroeder (1967). The resin was equilibrated in aqueous solution containing 1% pyridine-picoline-lutidine, and sufficient acetic acid to reach a pH 8.5 and packed into a 0.9×60 cm column. The glycopeptide was applied in the above buffer and elution was carried out using different buffers as indicated by arrows. Fractions of 2.1ml were collected at a flow rate of 45ml/h. Glycopeptides were detected by the phenol-H_2SO_4 acid reaction.

the recovered carbohydrate was: peak 1 (GP-C_{3a}), 83% and peak 2 (GP-C_{3b}), 17%. Table 1 gives the yield and total recovery of glycopeptides during each step of purification.

Properties of glycopeptides

Homogeneity: When subjected to electrophoresis at pHs 1.8, 3.4 and 6.4, the purified glycopeptides moved as single bands.

Molecular weight: A plot of log molecular weight against Ve/Vo of the standards is shown in figure 4. From the graph, the molecular weights calculated for glycopeptides GP-C_1, GP-C_2, GP-C_{3a}, GP-C_{3b}, GP-D_1 and GP-D_2 were 3600, 3900, 3500, 3700, 2750 and 1600 respectively.

Table 1. Yield and total recovery of glycopeptides.

Fraction	Yield (mg)	Overall recovery[a] %
Pronase digest (from 1.4 g of glucoamylase II)	250	100
GP-A (Biogel P-30)	212	85
GP-B (Biogel P-30)	200	80
High voltage electrophoresis		
GP-C	145	70
GP-D	30	
Dowex-50X2 chromatography		
GP-C_1	35	52
GP-C_2	30	
GP-C_3	40	
GP-D_1	20	
GP-D_2	5	
Dowex-1X2 chromatography		
GP-C_1	30	42
GP-C_2	25	
GP-C_{3a}	25	
GP-C_{3b}	5	
GP-D_1	16	
GP-D_2	4	

[a] As glucose equivalent.

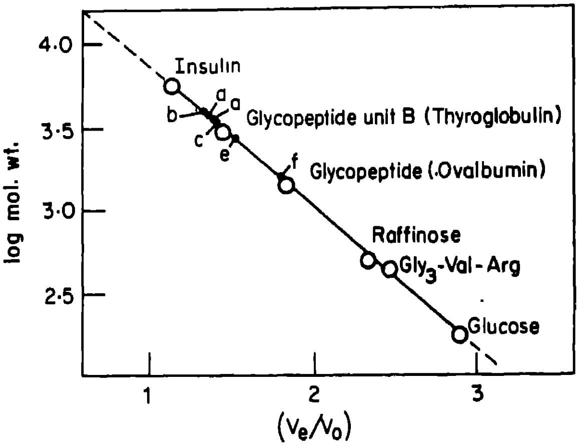

Figure 4. Molecular weight determination of glycopeptides on Biogel P-10.

Experimental details are given in Materials and Methods. Ve, elution volume; Vo, void volume. a, GP-C; b, GP-C_2; c, GP-C_{3a}; d, GP-C_{3b}; e, GP-D_1; f, GP-D_2.

The molecular weight of two glycopeptides namely GP-C_2 and GP-C_{3a}, was also determined by the Archibald method. Values of 4000 (±300) and 3800 (±200) were obtained respectively. A partial specific volume of 0.664 ml/g was employed in the molecular weight calculations.

Amino-terminal end group: Serine was found to be the N-terminal amino acid in all the glycopeptides. Traces of glycine and alanine were also seen on the chromatograms.

Amino acid composition: The amino acid composition of purified glycopeptides is given in Table 2. The amino acids serine and threonine were the major components and proline, glycine and alanine were the minor ones in all of the isolated glycopeptides. Small amounts of tyrosine were also present in GP-D glycopeptides. The ratio of threonine to serine was 2.3 to 2.4 in GP-C glycopeptides, but was unity in GP-D glycopeptides. The number of residues of different amino acids present in each glycopeptides based on molecular weights determined by gel filtration are also given in Table 2. Most of the amino acids, including minor ones, occurred in non-integral molar ratios, perhaps due to destruction during hydrolysis; in some instance, less than one residue was obtained. Treatment of each glycopeptide with pronase caused no change in the amino acid composition. It is likely therefore that pronase attacks different peptide bonds at different rates, producing a mixture of glycopeptides, each of which is no longer susceptible to pronase action.

Table 2. Amino acid and carbohydrate composition of the glycopeptides.

Components	GP-C_1 mols/mol	GP-C_2 mols/mol	Gp-C_{3a} mols/mol	GP-C_{3b} mols/mol	GP-D_1 mols/mol	GP-D_2 mols/mol
Amino acids						
Thr[a]	6.68(7)*	6.63(7)	5.77(6)	5.6(6)	3.98(4)	3.68(4)
Ser[a]	2.88(3)	2.73(3)	2.55(3)	2.62(3)	3.70(4)	3.52(4)
Pro	Traces	0.50(1)	0.50(1)	0.59(1)	0.68(1)	0.61(1)
Gly	0.54(1)	0.82(1)	0.42	0.66(1)	Traces	0.52(1)
Ala	Traces	0.74(1)	0.32	0.70(1)	Traces	0.19
Tyr					0.19	0.19
Sugars						
Man[b]	11.5(12)	14.5(15)	10.5(11)	10.36(10)	7.98(8)	3.68(4)
Glc[b]	1.18(1)	0.7(1)	0.7(1)	1.1(1)	0.96(1)	
Gal[b]	1.18(1)	0.66(1)			0.82(1)	0.51(1)
Xyl[b]					0.96(1)	0.31
GlcN[c]			2.5(3)	2.6(3)		

[a] Uncorrected for destruction

[b] By gas liquid cromatography as alditol acetates

[c] By the Elson-Morgan reaction

* Figures in parentheses have been rounded off to the nearest integar.

Carbohydrate composition: The sugar composition determined for these glycopeptides is also given in table 2. On this basis glycopeptides appeared to belong to three distinct types: Type 1 glycopeptides, GP-C_1 and GP-C_2, contained mannose, glucose and galactose in the molar proportion 12-15:1:1 but not glucosamine or xylose. Type 2 glycopeptides, GP-C_{3a} and GP-C_{3b}, contained mannose, glucose and glucosamine in the molar proportion 10-11:1:2-3, but not galactose or xylose. Type 3 glycopeptides, GP-D_1 and GP-D_2, contained mannose, glucose, galactose and xylose in the molar proportion 3.7-8:0-1:0.5-0.8:0.3-1 but not glucosamine. In most of the isolated glycopeptides, each constituent sugar was present in a non-integral ratio.

Discussion

The glucoamylase II of *A. niger* contained 18% carbohydrate and its molecular weight was 72,000 (Manjunath and Raghavendra Rao, 1979). It contained, per mole, 45 residues of mannose, 3 of galactose, 4 of glucose, 2 of xylose and 3 of glucosamine. This is one of the many instances of glycoproteins containing so many different monosaccharides.

In order to investigate the carbohydrate moiety in the glycoprotein molecule, a convenient approach is to proteolyse the glycoprotein to a number of smaller fragments. The extent of proteolysis is likely to depend upon the size and the number of carbohydrate moieties connected to the peptide backbone, and also upon the protease used. In the present investigation, native glucoamylase II was not amenable to extensive pronase digestion: about 60% activity remained after two prolonged digestions, and the residual enzyme had a molecular weight of around 60,000. However, the denatured enzyme is susceptible to pronase action and yielded, after exhaustive digestion, four major glycopeptides that were purified to homogeneity by a combination of separation methods. The yield of the homogeneous glycopeptides was 42% of the original carbohydrate in the glycoprotein, which is fairly satisfactory. In the case of glucoamylase from *R. javanicus,* Watanabe and Fukimbara, (1973) obtained a 65% yield of glycopeptide. In the case of ovalbumin (Marshall and Neuberger, 1972) and α-amylase of *A. oryzae* (McKelvy and Lee, 1969) the yields of glycopeptides were reported to be around 80-90%.

The glycopeptides varied in their molecular weights from 1600 to 4000 and in the carbohydrate content from 50 to 70%. In *R. javanicus,* two glycopeptides had asparagine and either threonine or glycine as the amino acid components, whereas the third one had four amino acids (threonine, serine, alanine and proline) in addition to 60% carbohydrate (Watanabe and Fukimbara, 1974a.and 1974b). Such patterns of amino acid composition have been attributed to the use of a protease such as pronase with broad specificity (Watanabe and Fukimbara, 1973; Yamauchi, *et al.,* 1968; Spiro, 1962). Apparently pronase digestion proceeds to a different degree and rate in each case: perhaps the carbohydrate moieties and/or the amino acid sequence affect the extent of digestion.

The carbohydrate compositions of the glycopeptides generally differed from one another and also from that of the glycoprotein itself. All the six glycopeptides contained mannose and glucose and were grouped into three types on the basis of the monosaccharide present: one containing galactose; the second containing glucosamine instead of galactose; and the third with galactose and xylose. Further, in most of these glyco-peptides, the constituent sugars occured in non-integral ratios. For example, the glycopeptide GP-D_2 contained 0.33 mole of xylose and 0.51 mole of galactose per mole. Each glycopeptide fraction therefore presumably represented a family of extremely similar molecules all of broadly the same chemical composition and differing only slightly in carbohydrate composition. Since the compositional variations within each glycopeptide fraction were apparently rather small, the fraction, though not entirely homogeneous, behaved as a single compound during column chromatography and electrophoresis. Such "microheterogeneity" was reported to be a common feature of glycoproteins (Spiro, 1970; Sharon, 1974) particularly ovalbumin and α-amylase of *A. oryzae.*

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Studies on carbohydrate moieties of the glycoprotein, glucoamylase II of *Aspergillus niger:* Nature of carbohydrate-peptide linkage and structure of oligosaccharides

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Abstract. Electrophoretically homogeneous type 1 (GP-C$_1$ and GP-C$_2$), type 2 (GP-C$_{3a}$ and GP-C$_{3b}$) and type 3 (GP-D$_1$ and GP-D$_2$) glycopeptides from *Aspergillus niger* glucoamylase II (Manjunath and Raghavendra Rao, preceding paper) were separately treated with alkaline borohydride. The β-eliminated oligosaccharides were subjected to single and sequential digestion with specific glycosidases and the products analysed by gas liquid chromatography. The studies revealed that carbohydrate moieties were present as mannose, Man-Man-, and trisaccharide structures, namely, (a) Glc-Man-Man-, (b) Gal-Man-Man, (c) Man-Man-Man-, (d) GlcNAc-Man-Man-, and (e) Xyl-Man-Man. None of the glycopeptides contained all the trisaccharide structures (a) to (e). Type 1 glycopeptide contained structures (a), (b) and (c); type 2, (a) and (d) and type 3, (a), (b) and (e). The number of carbohydrate units (mono-, di- and trisaccharides) present in the major glycopeptides was determined and tentative structures for the glycopeptides proposed. Carbohydrate units appeared to occur in clusters of 4 to 7 in each glycopeptide, a structure unique to the carbohydrate moiety in *Aspergillus niger* glucoamylase. Based on carbohydrate analysis and yields of glycopeptide, the number of units of each type of glycopeptide present in glucoamylase II was tentatively calculated to give two of type Man:Glc:Gal=12-15:1:1, one of type Man:Glc:GlcN=10-11:1:2 and one of type Man:Glc:Gal:Xyl=4-8:0.1:0.5-0.8:0.3-1 glycopeptides.

Keywords. Glucoamylase; *Aspergillus niger;* glycopeptide; carbohydrate-peptide linkage; central heterogeneity.

Introduction

The isolation and purification of six glycopeptides viz., GP-C , GP-C$_2$, GP-C$_{3a}$, GP-C$_{3b}$, GP-D and GP-D$_2$ have been described and their molecular weight and composition has been indicated (Manjunath and Raghavendra Rao, preceding paper). All of them contained mannose and glucose besides other monosaccharides. Based on their content of other monosaccharides, the glycopeptides were grouped into three types: GP-C$_1$ and GP-C$_2$ forming one type, contained galactose, GP-C$_{3a}$ and GP-C$_{3b}$ forming another type, contained galactosamine, and GP-D$_1$ and GP-D$_2$ forming the third, contained galactose and xylose.

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The present investigation describes the results of experiments conducted to determine the structure of the carbohydrate moieties, the nature of the bonds linking them to peptide and the probable distribution of these units in glycopeptides.

Materials and methods

D-Galactose oxidase (EC 1.1.3.9), peroxidase (EC 1.11.1.7), jack-bean meal and *o*-dianisidine were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. β-Galactosidase (EC 3.2.1.23) (*Escherichia coli*) was from Worthington Biochemical Corporation, Freehold, New Jersey, USA. Sources of the chemicals have been described previously (Manjunath and Raghavendra Rao, previous paper).

Glycopeptides GP-C_1, GP-C_2, GP-C_{3a}, GP-C_{3b}, GP-D_1 and GP-D_2 were purified from the pronase digest of glucoamylase II of *A. niger* as described (Manjunath and Raghavendra Rao, previous paper).

Glycosidase preparation and activity

β-Galactosidase was purchased from the Worthington Biochemicals Corporation, Freehold, New Jersey, USA. α-Mannosidase (EC 3.2.1.24) and β-N-acetyl glucosaminidase (EC 3.2.1.30) were purified from Jack bean meal (from Sigma Chemical Co., St. Louis, Missouri, USA) according to the procedure of Li (1966 and 1967). α-Mannosidase and β-N-acetyl glucosaminidase thus prepared were free from each other and also from β-galactosidase and β-xylosidase. An aqueous extract of Jack bean meal was found to contain β-xylosidase (EC 3.2.1.37) and was used as such. Amyloglucosidase (glucoamylase) was purified as described earlier (Manjunath and Raghavendra Rao, 1979). The assay conditions, substrates and specific activities for these enzymes used in the present investigation are indicated in table 1.

Table 1. Specific activities of glycosidases used*

Enzyme	Substrate	Specific activity
α-Mannosidase[a] (JBM)†	methyl-α-D-glucopyranoside	5.0
β-N-Acetyl glucosaminidase[b] (JBM)	ovalbumin glycopeptides	0.2
β-Galactosidase[c] (*E. coli*)	p-nitrophenyl-β-D-galactopyranoside	330.0
β-Xylosidase[d] (JBM)	*o*-nitrophenyl-β-D-xyloside	0.01
Glucoamylase[e] (*A. niger*)	Soluble starch	90.0

* All specific activity units are expressed as μ mols of product liberated/mg protein/min at 37°C in 0.5 M acetate buffer pH 4.6. Activity measured on the basis of the release of

[a] mannose
[b] N-acetylglucosamine
[c] and [d] p-nitrophenol
[e] Glucose
† JBM—Jack Bean Meal

Preparation of reduced oligosaccharides

Glycopeptides were treated with alkaline borohydride according to Carlson (Carlson, 1966) with the following modification. Freeze-dried glycopeptides (2-3 µmol) were treated with 10 ml of 0.1 M NaOH containing 1 M $NaBH_4$ at 37°C. After 50 h, the excess of borohydride was destroyed with dilute acetic acid (to pH 5) and concentrated. Borate was removed by repeated evaporations with methanol. The peptide-free material, obtained by passage through a column of Dowex-50×2 (H^+), was subjected to preparative paper chromatography (n-butanol:ethanol: water=10:1:2) and the various mono-, di and trisaccharides were isolated.

The peptide portion was eluted from the Dowex column with 1 M pyridine acetate (pH 5.0), evaporated to dryness and subjected to amino acid analysis as described earlier (Manjunath and Raghavendra Rao, previous paper).

Determination of terminal non-reducing monosaccharide residue using glycosidases.

The following procedure was used to detect terminal non-reducing sugars. To 1 to 2 µmol of oligosaccharide in 0.05 M acetate buffer pH 4.6 was added β-galactosidase (3 units), β-N-acetyl glucosamidase (8 units), α-mannosidase (15 units), glucoamylase (10 units) or β-xylosidase (0.1 unit). The reaction mixture (0.5 ml) was incubated for 30 to 48 h at 37°C with a few drops of toluene, then heated in a boiling water-bath for 3 min and centrifuged to remove the precipitated enzyme protein. The supernatant was then applied to a column (0.5×5 cm) of Dowex-50× 2(H^+) and washed with water. The washings were concentrated and examined by paper chromatography. The oligosaccharides eluted from the paper were evaporated to dryness, redissolved in 0.05 M acetate buffer pH 4.6 and incubated with another glycosidase. Controls consisting only of substrate or the enzyme accompanied each experiment. The released sugars were determined by gas liquid chromatography as described earlier (Manjunath and Raghavendra Rao, 1979). Glucose was also determined using tris glucose oxidase reagent. N-Acetyl glucosamine was determined by the method of Reissig *et al.* (1955), following the modification of Marshall and Neurber (1972). The presence of non-reducing (i.e., terminal) residues of D-galactose in the oligosaccharides was also detected using D-galactose oxidase following the procedure of Dawson and Clamp (1968).

Results

Structure of type 1 glycopeptides (GP-C_1 and GP-C_2)

The glycopeptides GP-C_1 and GP-C_2 were shown to contain threonine, serine, glycine, mannose, glucose and galactose in a molar ratio of approximately 7:3:1:12:1:1 (Manjunath and Raghavendra Rao, previous paper). In addition, GP-C_2 also contained one residue each of alanine and proline and 3 more residues of mannose. Fractional amounts of other amino acids were also present. The calculated molecular weights were 3320 and 3900 for GP-C_1 and GP-C_2 respectively. These values are in reasonable agreement with a value of 3600 and 3950 obtained by gel filtration.

Structure of glycopeptide GP-C_1: The glycopeptide GP-C_1 (10 mg=3 μmols) was subjected to alkaline borohydride treatment and mono-, di- and trisaccharides were isolated by paper chromatography as described in "Materials and methods".

The sugar components were examined by paper chromatography and quantitated by gas liquid chromatography. The results obtained are shown in table 2. High recoveries of glucose and galactose were obtained following alkaline borohydride treatment, Dowex-50 (H^+) column chromatography and paper chromatography and hydrolysis. The recovery of mannose was only 40% but most of the undetected mannose was recovered as mannitol. Mannitol was formed in the system from mannose units linked by alkali-sensitive glycopeptide bonds. On a molar basis approximately 7 mol of mannitol were formed per mol of glycopeptide. On the basis of yield of saccharides and their reduced products, there were approximately 2 monosaccharide units, 3 disaccharide units and 2 trisaccharide units per mole of glycopeptide.

Table 2. Molar ratios of sugar recoveries from alkaline borohydride treated glycopeptide GP-C_1.

Fraction	Mannose[a]	Mannitol[b]	Glucose[a]	Galactose[a]
1. GP-C_1	12	—	1	1
2. Dowex-50 eluate	5	7	1	1
3. Paper Chromatography				
Monosaccharide	—	2	—	—
Disaccharide	3	3	—	—
Trisaccharide	2	2	1	1

[a] Analyzed by gas liquid chromatography as alditol acetates

[b] Determined by gas liquid chromatography after separation from mannose by paper chromatography using solvent 1-butanol-pyridine H_2O, 6:4:3(v/v).

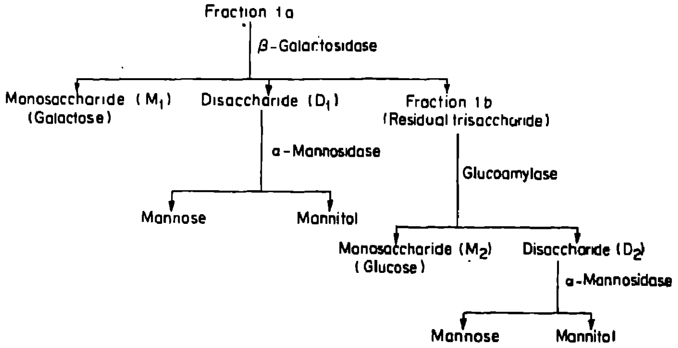
— means not present.

Structure of disaccharides: Upon treatment with α-mannosidase the disaccharide was cleaved into mannose and mannitol. Therefore the disaccharide was α-Man-Man-OH.

Structure of trisaccharides: Sugar composition of the trisaccharide fraction designated as fraction 1a indicated the presence of mannitol, mannose, glucose and galactose in the molar ratios 2:2:1:1. Hence fraction 1a should be a mixture of trisaccharides of differing composition.

The structure of the trisaccharides in fraction 1a was determined as follows: Fraction 1a was treated successively with various glycosidases as shown in figure 1

Figure 1. Scheme for the action of glycosidases on fraction 1a derived from GP-C_1.



and the released sugars were quantitatively determined at each step. The results obtained in such studies are shown in table 3. From these results it was deduced that two trisaccharides with the possible structure Glc-Man-Man-OH and Gal-Man-Man-OH were present.

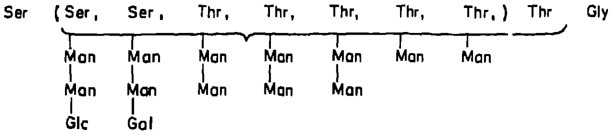
Table 3. Sugar composition of fraction 1a and its derivatives.

Fraction	Mannose	Mannitol	Glucose	Galactose (mols./mol glycopeptide)
Fraction 1a (original)	1.8	1.78	0.86	0.87
Fraction M_1	—	—	—	0.82
Fraction D_1	0.85	0.85	—	—
Fraction 1b	0.8	0.82	0.8	—
Fraction M_2	—	—	0.8	—
Fraction D_2	0.78	0.78	—	—

Monosaccharides were quantitated by gas liquid chromatography as alditol acetates. M_1 and other fractions were obtained from 1a by the procedure in figure 1. — = not present.

Amino acid composition: The amino acid composition both before and after treatment with alkaline borohydride was determined (table 4). Approximately 5 residues of Thr and 2 residues of Ser were lost per mole of glycopeptide. The combined loss of threonine and serine was found to be equivalent to the mannitol formed in the system. These results therefore suggested that there are seven mannose residues in O-glycosidic linkages with 5 residues of Thr and 2 of Ser.

From the data obtained, it may be inferred that GP-C_1 contained (per mole) 2 units of Thr/Ser-Man, 3 units of Thr/Ser-Man-Man, 1 unit of Thr/Ser-Man-Man-Gal and 1 unit of Thr/Ser-Man-Man-Glc. The sequence of amino acids in glycopeptide was not determined. A tentative structure (I) is proposed for GP-C_1 on the basis of the facts now known:


Ser
(Ser, Ser, Thr, Thr, Thr, Thr, Thr,) Thr Gly
Man Man Man Man Man Man Man
Man Man Man Man Man
Glc Gal

(I)

The saccharides could be present in a different order from that shown.

Structure of glycopeptide GP-C_2: This glycopeptide (10 mg) when subjected to alkaline borohydride treatment yielded di- and trisaccharides. These saccharides were isolated and their composition determined as described above for GP-C_1.

Table 4. Effect of alkaline borohydride treatment on amino acid composition of GP-C_1.

Component	Original	After alkaline borohydride treatment
Threonine[a]	7	2
Serine[a]	3	1
Glycine	1	1
Alanine	—	2
α-amino-butyrate	—	1

[a] Values not corrected for any destruction due to acid hydrolysis.

The results obtained are shown in table 5. Nearly 7 moles of mannose of a total of 15 disappeared and most of it was recovered as mannitol. There were approximately 4 units of disaccharides and 3 units of trisaccharides.

Structure of disaccharide: The disaccharide was cleaved by α-mannosidase to give mannose and mannitol in the ratio 1:1. Hence the disaccharide has a Man-Man-OH structure.

Structure of trisaccharides: The trisaccharide fraction designated fraction 2a was found to contain Man-OH, Man, Glc and Gal in the ratio 4.7:7.1:1:1 (table 5). Therefore fraction 2a is clearly a heterogeneous mixture of trisaccharides. The trisaccharide structures in fraction 2a were determined as follows:

Table 5. Molar ratios of sugar recoveries from alkaline-borohydride treated glycopeptide GP-C_2

Fraction	Mannose	Mannitol	Glucose	Galactose
1. GP-C_2	15	—	1	1
2. Dowex-50	8	7	1	1
3. Paper chromatography				
Disaccharide	4	4	—	—
Trisaccharide	4	3	1	1

Experimental conditions as in Table 2. — = not present.

Fraction 2a was treated with various glycosidases as shown in figure 2 and the released sugars were quantitatively determined at each step. The results obtained by such studies are shown in table 6. From these results it is possible to deduce the structures of three trisaccharides, namely Man-Man-Man-OH, Gal-Man-Man-OH and Glc-Man-Man-OH.

Figure 2. Scheme for the action of glycosidases on fraction 2a derived from GP-C_2.

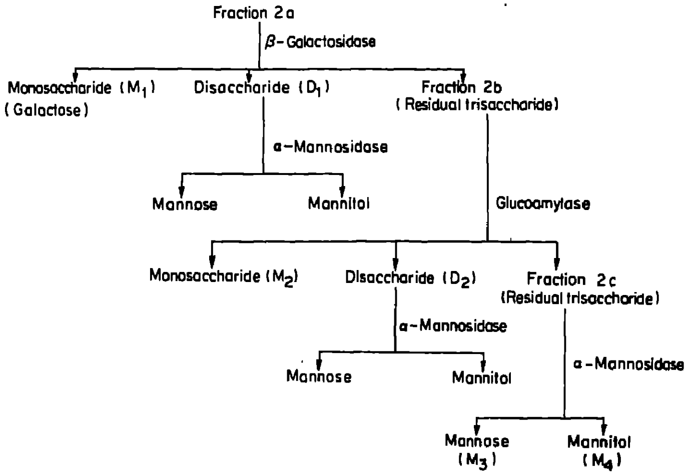


Table 6. Sugar composition of fraction 2a and its derivatives.

Fraction	Mannose	Mannitol	Glucose	Galactose
		(mol./mol. of glycopeptide)		
2a	4.00	2.64	0.60	0.56
M_1	—	—	—	0.50
D_1	0.50	0.50	—	—
2b	3.38	2.00	0.56	—
M_2	—	—	0.52	—
D_2	0.50	0.50	—	—
2c	2.78	1.40	—	—
M_3	2.70	—	—	—
M_4	—	1.30	—	—

Fractions mentioned were obtained from 2a by the procedure in figure 2. — = not present.

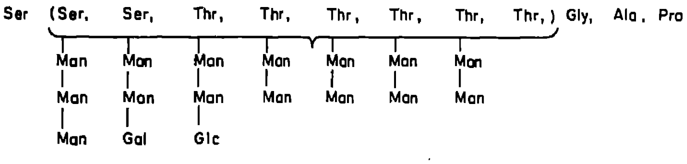
Amino acid composition: Amino acid composition of glycopeptide GP-C_2 before and after alkaline borohydride treatment is given in table 7. Nearly 5 mol of threonine and 2 mol of serine were destroyed and this was almost equal to the mannitol formed in the system. Therefore 7 residues of mannitol are involved in 0-glycosidic linkage with 5 residues of threonine and 2 residues of serine.

The data for GP-C_2 show that it contained per mol, 4 units of Thr/Ser-Man-Man, 1 unit of Thr/Ser-Man-Man-Man, 1 unit of Thr/Ser-Man-Man-Glc and 1 unit of Thr/Ser-Man-Man-Gal. Based on these data, a tentative structure (II) is proposed forGP-C_2.

Table 7. Effect of alkaline borohydride treatment on amino acid composition of GP-C_2.

Component	Original (mol./mol.)	After alkaline borohydride treatment
Threonine[a]	7	2
Serine[a]	3	1
Proline	1	1
Glycine	1	1
Alanine	1	2
α-amino-butyrate	—	1

[a] Values not corrected for any destruction by acid hydrolysis. — = not present.


Ser (Ser, Ser, Thr, Thr, Thr, Thr, Thr, Thr,) Gly, Ala, Pro
Man Man Man Man Man Man Man
Man Man Man Man Man Man Man
Man Gal Glc

(II)

Structure of type 2 glycopeptides (GP-C_{3a} and GP-C_{3b})

Glycopeptide GP-C_{3a} has been shown to contain threonine, serine, proline, mannose, glucose and glucosamine in the molar ratio 6:3:1:11:1:3 (Manjunath and Raghavendra Rao, previous paper). Glycopeptide GP-C_{3b} had the same composition but one residue less of mannose. In addition, GP-C_{3b} also contained one mole each of glycine and alanine. The calculated molecular weights for GP-C_{3a} and GP-C_{3b} were 3500 and 3480, which were in good agreement with the values of 3500 and 3700 obtained by gel filtration.

Structure of glycopeptide GP-C_{3a}: This glycopeptide yielded mono-, di- and trisaccharides on treatment with alkaline borohydride. The saccharides were separated and their composition determined as before. The results are shown in table 8. Approximately 6 mol of mannitol were formed in the system indicating mannose to be linked with the peptide chain.

Structure of disaccharide: The disaccharide had a Man-Man-OH structure since it was cleaved by α-mannosidase.

Structure of trisaccharides: The trisaccharide fraction designated as fraction 3a contained Man, Glc, GlcN and Man-OH, and hence was heterogeneous. The structure of trisaccharides in fraction 3a was determined following the scheme shown in figure 3. The results obtained are indicated in table 9. From these results two trisaccharides were deduced to occur, namely Glc-Man-Man-OH and GlcNAc-Man-Man-OH.

Table 8. Molar ratios of sugar recoveries from alkaline-borohydride treated glycopeptide GP-C_{3a}.

Fraction	Mannose	Mannitol	Glucose	Glucosamine[a]
			(mols/mol)	
1. GP-C_{3a}	11	—	1	3
2. Dowex-50 eluate	5	5	1	3
3. Paper Chromatography				
Monosaccharide	—	1	—	—
Disaccharide	1	1	—	—
Trisaccharide	3	3	1	2-3

Experimental conditions as in table 2.

[a] Determined as N-acetyle glucosamine by Reissig *et al.* (1955).

Figure 3. Scheme for the action of glycosidases on fraction 3a and its derivatives.

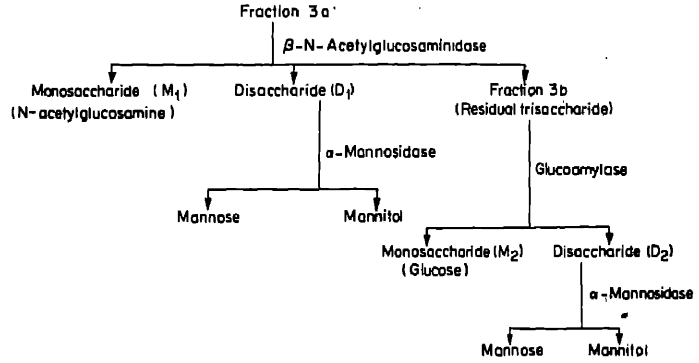


Table 9. The composition of fraction 3a and its derivatives.

Fraction	Mannose	Mannitol	Glucose	N-acetyl-Glucosamine
			(mol./mol. of glycopeptide)	
3a	3.48	3.48	0.72	2.72
M_1	—	—	—	2.60
D_1	2.63	2.63	—	—
3b	0.65	0.65	0.65	—
M_2	—	—	0.60	—
D_2	0.60	0.60	—	—

The fractions were obtained from 3a by the procedure in figure 3. GlcNAc was determined according to Reissig *et al.* (1955).

Amino acid composition: Glycopeptide GP-C_{3a} after treatment with alkaline borohydride resulted in the loss of approximately 4 residues of threonine and 2 residues of serine (table 10). These are therefore involved in the linkage.

Data obtained for GP-C_{3a} suggest that it contained approximately 1 unit of Thr/Ser-Man, 1 unit of Thr/Ser-Man-Man, 1 unit of Thr/Ser-Man-Man-Glc and 3 units of Thr/Ser-Man-Man-GlcNAC. Based on these results, a tentative structure (III) is proposed for this glycopeptide.

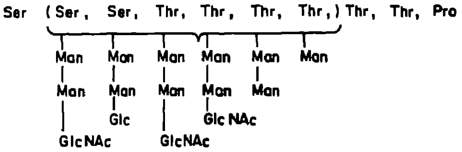


Table 10. Effect of alkaline borohydride treatment on amino acid composition of GP-C_{3a}.

Component	Original	After alkaline borohydride treatment
	(mols/mol)	
Threonine[a]	6	2
Serine[a]	3	1
Proline	1	1
Alanine	–	2
Glycine	—	—
α-amino-butyrate	—	1

[a] Values not corrected for any destruction by acid hydrolysis.

Structure of glycopeptide GP-C_{3b}: Because of low yields of glycopeptide GP-C_{3b}, no quantitative data could be obtained on this glycopeptide. However, some qualitative results were obtained.

Treatment of GP-C_{3b} with alkaline borohydride eliminated all the carbohydrate from the glycopeptide. This also resulted in the loss of nearly 60-70% of both serine and threonine. Mannitol was detected in the system. These results therefore indicated the occurence of O-glycosidic linkages involving threonine and serine with mannose. From the β-eliminated carbohydrate fraction mono- and trisaccharide fractions were isolated. The trisaccharide fraction, on treatment with β-N-acetylglucosaminidase, released N-acetyl-glucosamine. Therefore trisaccharide structures similar to those present in GP-C_{3a} seem to be present in glycopeptide GP-C_{3b} also. It appears reasonable to conclude that the glycopeptide GP-C_{3b} has a structure similar to GP-C_{3a}, but differs from it since it contained an additional residue each of alanine and glycine.

Structure of type 3 glycopeptides (GP-D_1 and GP-D_2)

Glycopeptide GP-D_1: Glycopeptide GP-D_1 was shown to contain threonine, serine, proline, mannose, glucose, galactose and xylose in the molar ratio approximately 4:4:1:8:1:1:1 (Manjunath and Raghavendra Rao, previous paper).

Fractional amounts of other amino acids were also present. This glycopeptide yielded only di- and trisaccharides on treatment with alkaline borohydride. The compositions of these fractions are shown in the table 11. Approximately 4 mol of mannitol were formed in the system indicating mannose to be involved with the peptide chain.

Structure of disaccharide: As before, the disaccharide was identified as Man-Man-OH.

Table 11. Molar ratios of sugar recoveries from alkaline borohydride treated glycopeptide GP-D_1

Fraction	Mannose	Mannitol	Glucose	Galactose	Xylose
GP-D_1	8	–	1	1	1
Dowex-50	4	4	1	1	1
Paper Chromatography Disaccharide	1	1	–	–	–
Trisaccharide	3	3	1	1	1

Experimental conditions as in Table 2. Monosaccharides and derivatives were determined as alditol acetates.

Structure of trisaccharide: The trisaccharide fraction (designated fraction 4a) consisted of mannose, mannitol, glucose, galactose and xylose and was hence heterogeneous. The structures of the trisaccharides in fraction 4a were established following the scheme shown in figure 4 and the results obtained are shown in table 12. From these results, structures of three trisaccharides were deduced as Gal-Man-Man-OH, Glc-Man-Man-OH and Xyl-Man-Man-OH.

Figure 4. Scheme for the action of glycosidases on fraction 4a and its derivatives.

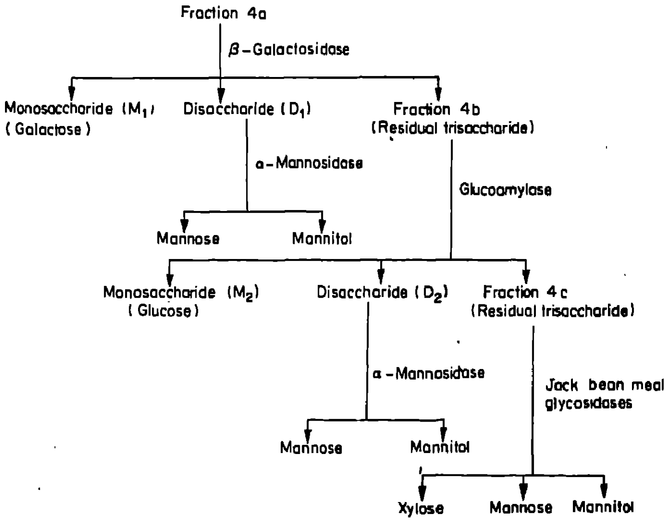

Fraction 4a
β-Galactosidase
Monosaccharide (M1) (Galactose)
Disaccharide (D1)
Fraction 4b (Residual trisaccharide)
α-Mannosidase
Mannose
Mannitol
Glucoamylase
Monosaccharide (M2) (Glucose)
Disaccharide (D2)
Fraction 4c (Residual trisaccharide)
α-Mannosidase
Mannose
Mannitol
Jack bean meal glycosidases
Xylose
Mannose
Mannitol

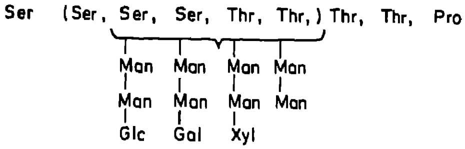
Table 12. Sugar composition of fraction 4a and its derivatives.

Fraction	Mannose	Mannitol	Glucose	Galactose	Xylose
			(mol./mol. of glycopeptide)		
4a	3.64	3.64	1.3	1.08	1.28
M_1	—	—	—	0.98	—
D_1	0.98	0.98	—	—	—
4b	2.50	2.48	1.2	—	1.20
M_2	—	—	1.2	—	—
D_2	1.20	1.20	—	—	—
4c	1.10	1.10	—	—	1.10
M_3	—	—	—	—	1.00
D_3	1.02	1.02	—	—	—

The fractions were obtained from 4a by the procedure in figure 4.

Amino acid composition: Amino acid composition after treatment with alkaline borohydride is shown in Table 13. Approximately 2 residues each of threonine and serine disappeared during the treatment and these are therefore involved in the O-glycosidic linkage. Therefore there is one unit each of Thr/Ser-Man-Man, Thr/Ser-Man-Man-Glc, Thr/Ser-Man-Man-Gal and Thr/Ser-Man-Man-Xyl.

On the basis of these results a tentative structure (IV) is proposed for GP-D_1



(IV)

Table 13. Effect of alkaline borohydride treatment on amino acid composition of GP-D_1.

Component	Original	After alkaline borohydride treatment
	(mol./mol.)	
Threonine[a]	4	2
Serine[a]	4	2
Proline	1	1
Glycine	—	—
Alanine	—	1
α-amino-butyrate	—	1
Tyrosine	—	—

[a] Values uncorrected for any destruction by acid hydrolysis.

Glycopeptide GP-D_2: The very limited amount of GP-D_2 available did not permit detailed investigations on this glycopeptide. On chemical analysis, this glycopeptide showed mannitol, galactose, threonine, serine, proline, glycine and xylose in the molar ratio 4:1:4:4:1:1:0.3. Treatment of GP-D_2 with alkaline borohydride eliminated all the carbohydrate, besides resulting in the loss of nearly 45-50% of both threonine and serine. Mannitol was detected in the system. These results again indicated an O-glycosidic linkage similar to GP-D_1. The β-eliminated product when chromatographed on paper gave spots corresponding to mono-, di- and trisaccharides. The monosaccharide was identified as mannitol and the disaccharide as Man-Man-OH. The trisaccharide fraction contained mannitol, mannose, galactose, traces of glucose and xylose indicating heterogeneity of the trisaccharide. On the basis of these data it may be inferred that GP-D_2 has a structure similar to GP-D_1.

Discussion

The present study was directed at examining the nature of linkage between carbohydrate and peptide moieties, and the structure of the oligosaccharide, following which an average structure for each glycopeptide has been proposed. Four of the six glycopeptides isolated, namely GP-C_1, GP-C_2, GP-C_{3a} and GP-D_1 were obtained in substantial quantity. Only these glycopeptides were investigated in detail. Although no extensive studies were made on GP-C_{3b} and GP-D_2, the available data indicate that GP-C_{3b} has a structure similar to GP-C_{3a} and GP-D_2 was similar to GP-D_1. No work on the sequence of amino acids in the glycopeptides except the determination of the N-terminal amino acids has been conducted.

Nature of linkage

When the glycopeptides were treated with alkaline borohydride, a decrease of serine and threonine with a simultaneous increase in alanine and *de novo* appearance of α-amino butyric acid and mannitol was noted with all the glycopeptides. Besides, mannitol was the only sugar alcohol obtained. Clearly the carbohydrate moieties in all the glycopeptides are O-glycosidically linked through mannose to the hydroxyls of serine and threonine. However, the number of such linkages differed for each type of glycopeptide. Thus type 1, type 2 and type 3 glycopeptides contained 7, 6 and 4 O-glycosidic linkages, respectively. In all of them, two linkages involved serine and mannose and the rest involved threonine and mannose.

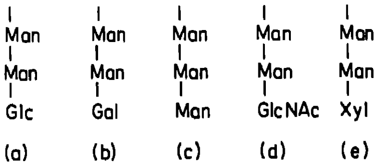
The presence of N-acetylglucosamine might indicate a linkage with asparagine. However there was no trace of aspartic acid in the acid hydrolyzate of the glycopeptides. Besides, the GlcNAc-Asn linkage is resistant to alkaline borohydride (Spiro, 1966). Secondly, the trisaccharides obtained by β-elimination did not contain N-acetyl glucosaminitol but only N-acetylglucosamine which could be released from the trisaccharides by N-acetylglucosaminidase. These facts ruled out any direct linkage of N-acetylglucosamine with the peptide backbone in the present instances. Therefore the linkage of the carbohydrate to the peptide chain seemed to be exclusively through the O-glycosidic bonds with the aliphatic hydroxy amino acids. These results in general are in conformity with those of Lineback *et al.* (1972) and Pazur *et al.* (1971) on the glucoamylases of *A. niger* and

A. foetidus respectively. However their results were based on alkaline borohydride treatment of glucoamylase itself and not the isolated glycopeptides.

The glycopeptides of glucoamylase of *R. javanicus* (Watanabe and Fukimbara, 1974a, b, and 1975; Watanabe, 1976) seem to be quite distinct from the ones described here. Two of them had GlcNAc-Asn bonds whereas the third glycopeptide had O-glycosidic bonds between aliphatic hydroxy amino acids and mannose. If this proves typical of the *Rhizopus* glucoamylases, then these differ very markedly from the enzyme from black *Aspergilli* in the linkage of the carbohydrates to the peptide backbone.

Structure of the Oligosaccharides and heterogeneity

Sequential digestion of the trisaccharides with specific glycosidases revealed at least 5 different trisaccharide structures:



$$\begin{array}{ccccc} | & | & | & | & | \\ \text{Man} & \text{Man} & \text{Man} & \text{Man} & \text{Man} \\ | & | & | & | & | \\ \text{Man} & \text{Man} & \text{Man} & \text{Man} & \text{Man} \\ | & | & | & | & | \\ \text{Glc} & \text{Gal} & \text{Man} & \text{GlcNAc} & \text{Xyl} \\ \text{(a)} & \text{(b)} & \text{(c)} & \text{(d)} & \text{(e)} \end{array}$$

(V)

None of the glycopeptides investigated here contained all the five trisaccharide structures (a) to (e). Type 1 glycopeptides contained structures (a), (b) and (c); type 2 glycopeptides contained structures (a) and (d), and type 3 glycopeptides contained structures (a), (b) and (e). Thus, the glycopeptides differ from one another in their trisaccharide structures. This type of heterogeneity has been described as 'central heterogeneity' (Clamp, *et al.*, 1968). In addition to trisaccharides, all the glycopeptides in the present investigation contained either mannose or Man-Man- or both in varying amounts. These are probably the products of incomplete glycosylation. Though a molar monosaccharide composition in each glycopeptide has been assigned, in most of the glycopeptides each constituent monosaccharide occurred in a non-integral (fractional) ratio. This has been termed "microheterogeneity" and has been discussed already (Manjunath and Raghavendra Rao, previous paper). Even the very simple Glc-Gal-disaccharide unit of collagens manifests "microheterogeneity" since units consisting only of the internal galactose occur in addition to the disaccharide (Spiro, 1969). The microheterogeneity observed in the carbohydrate structures of glycopeptides is consistent with the present concept of glycoprotein biosynthesis in which it is proposed that each sugar residue is added stepwise to a growing chain (Spiro, 1970 and 1973; Sharon, 1974). Thus the microheterogeneity found in so many glycoproteins might represent oligosaccharide chains in varying stages of completion, or resulting from transglycosylations that do not have an absolute specificity (Spiro, 1970 and 1973; Montgomery, 1972). With its large number of units, heterogeneity in glucoamylase may be even more apparent than in proteins with fewer units, for, the presence of multiple attachment sites along the peptide chain may lead to a greater number of sterically unfavourable conditions in which glycosylation may be impeded.

Two instances which have provided information on the structure of carbohydrate moieties of glycoamylases are due to Lineback *et al.*, (1972) and Watanabe and Fukimbara (1974a, b, 1974; Watanabe, 1976). Lineback *et al.*, (1972) isolated carbohydrate units after treating *A. foetidus* (Diazyme) glycoamylase I and glucoamylase II with alkaline borohydride and obtained trisaccharides and also a small fraction of mono- and disaccharides. They interpreted their results to mean that both glucoamylase I and glucoamylase II contained only trisaccharide structures of the types Man-Man-Man- and Man-Glc-Man- since they found that the trisaccharide structures were hydrolyzed partially to give mono- and disaccharide units. This apparently was due to the acid condition they used (0.1 N acetic acid) during the isolation of the oligosaccharides. In our experiments, it is quite unlikely that there is any hydrolysis of the trisaccharides because incubating isolated oligosaccharides or glycopeptides in 0.1 N acetic acid did not release any monosaccharides and therefore there was no cleavage of glycosidic linkage. Other trisaccharide structures involving galactose were also indicated by Lineback *et al.*, (1972) in their experiment and the trisaccharide units must therefore be heterogeneous in nature. One difference lies in the sequence of monosaccharides in the glucose containing trisaccharide which in the present studies is clearly shown to be Glc-Man-Man-. Moreover, the Diazyme glucoamylase did not contain trisaccharides containing Xyl and GlcNAc, since the native protein itself did not carry these sugars.

One of the three glycopeptides (GP-II) from *R. javanicus* glucoamylase contained six disaccharide units linked O-glycosidically to 5 threonine and 1 serine residues (Watanabe and Fukimbara, 1974a, 1974b and 1975). These glycopeptides bear an obvious resemblance to the ones described here. *R. javanicus* glycopeptide GP-II contained only Man-Man-disaccharides, whereas the present *A. niger* glucoamylase glycopeptides contained structures varying from monosaccharides to trisaccharides. Further, the trisaccharides in *A. niger* glucoamylase were heterogeneous.

On the other hand, the carbohydrate moiety of two other glycopeptides (GP-Ia and GP-Ib) of *R. javanicus* glucoamylase have a complex structure (Watanabe, and Fukimbara, 1974a, 1974b and 1975; Watanabe, 1976). In glycopeptides GP-Ia and GP-Ib the carbohydrate was shown to be present in the form of large and branched heterosaccharides. Carbohydrate-peptide link was through an N-glucosidic linkage between N-acetylglucosamine and asparate. The carbohydrate unit in this instance resembled that of ovalbumin (Montgomery, 1972; Marshall and Neuberger, 1970) or of α-amylase (Marshall and Neuberger, 1970). However such heteropolysaccharide structures were totally absent from *A. niger* glucoamylase.

Glycopeptide types in glucoamylase II

Although glucoamylase II contained a large number of glycopeptides which differed in the monosaccharide composition, an attempt was made to allocate carbohydrate units in terms of the relative abundance of each type of glycopeptide isolated. This might give us a picture of the number of mol of each glycopeptide per mole of glucoamylase II (table 14). Thus one mol of glucoamylase II appeared to contain an average two mols of type 1, one of type 2 and one of type 3 glyco-

Table 14. Glycopeptide units of glucoamylase II.

	Monosaccharide residues				
	Extrapolated from experimentally obtained data on resolved glycopeptides[d]				Present in original glycoprotein[e]
	GP-Type 1 2 units[a]	GP-Type 2 1 unit[b]	GP-Type 3 1 unit[c]	Total	Total
Mannose	26	11	8	45	45
Glucose	2	1	1	4	4
Galactose	2	0	1	3	3
Xylose	0	0	1	1	2
Glucosamine	0	3	0	3	3

The results are expressed as residues of monosaccharides experimentally found in each glycopeptide corrected to the nearest whole number, from which the sum of the residues in the postulated glycopeptide units has been totalled. This figure is compared with the number of residues experimentally found in the original glycoprotein.

GP, Glycopeptide

[a] Equal quantities of GP-C_1 and GP-C_2, hence total values employed.
[b] Almost 80-90% GP-C_{3a}, hence this value employed.
[c] Almost 80-90% GP-D_1, hence this value employed.
[d] Manjunath and Raghavendra Rao, previous paper.
[e] Manjunath and Raghavendra Rao, (1979).

peptide units. The number of mols of monosaccharide per mol of the glucoamylase calculated on the basis of the above allocation of glycopeptide units agreed very well with the analytical values for the same. The minor glycopeptides may perhaps replace the major units in a smaller number of protein molecules, and may represent incomplete or aberrant units.

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Alterations in the activities of the enzymes of proline metabolism in Ragi *(Eleusine coracana)* leaves during water stress

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Abstract. Free proline content in Ragi (*Eleusine coracana*) leaves increased markedly (6 to 85 fold) as the degree of water stress, created by polyethylene gylcol treatment, was prolonged. There was also a marginal increase in soluble proteins in the stressed leaves as compared to that in the controls. Water stress stimulated the activities of ornithine aminotransferase and pyrroline-5-carboxylate reductase, the enzymes of proline biosynthesis and markedly inhibited the enzymes involved in proline degradation viz., proline oxidase and pyrroline-5-carboxylate dehydrogenase. These results suggest that increase in free proline content of Ragi leaves could be due to enhanced activities of the enzymes synthesizing proline but more importantly due to severe inhibition of the enzymes degrading proline. These observations establish for the first time, the pathway of proline metabolism in plants by way of detection of the activities of all the enzymes involved and also highlight the role of these enzymes in proline accumulation during water stress.

Keywords. Proline; water stress; *Eleusine coracana;* ornithine aminotransferase; pyrroline-5-carboxylate reductase; proline oxidase; pyrroline-5-carboxylate dehydrogenase.

Introduction

A cascade of events, involving several biochemical changes is triggered by the withdrawal of water from plants. Although the early events following water stress are not clearly known, the most significant and immediately detectable change accompanying water stress is the accumulation of free proline. The free proline levels increase markedly in Bermuda grass and barley leaves during water deprivation (Barnett and Naylor, 1966; Kemble and Macpherson, 1954; Routley, 1966; Singh *et al.*, 1973). It has been shown that a few drought resistant crop varieties accumulate more proline as compared to the drought susceptible varieties (Srinivasa Reddy and Sastry, 1977). A relationship between yield stability index and proline accumulation has also been shown in barley genotypes (Singh *et al.*, 1972). These findings indicate that accumulation of proline may provide a biochemical adaptation for plants during water stress. Though several attempts have been made to establish a relationship between proline accumulation and drought tolerance, not much is known about the regulation of proline metabolism in plants grown under drought conditions. It is, therefore, of equal importance to study the regulation of this biochemical phenomenon in plants during water stress.

The biosynthesis and degradation of proline using radio-active precursors and detection of enzymes involved in these processes have been carried out in some detail (Boggess and Stewart, 1976; Boggess *et al.*, 1976; Wright *et al.*, 1977; Mazelis and Creveling, 1973; Boggess *et al.*, 1975; Mazelis and Fowden, 1969; Huber, 1974; Stewart *et al.*, 1977). However, all the enzymes involved in the biosynthesis and degradation of proline have not been reported in a single plant source and a comparative study of these enzymes have not been carried out in normal and water stressed plants.

With the objective of detecting enzymes involved in the conversion of ornithine to proline and exploring a cause-effect relationship between proline accumulation and drought resistance. Ragi (*Eleucine coracana*), a crop grown under rainfed conditions in Karnataka State,parts of which are drought-prone, was chosen for this study and the activities of the biosynthetic and degradative enzymes of proline metabolism were examined.

Materials and methods

Materials

All biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Polyethylene glycol (6,000 molecular weight) was obtained from SD's Lab Chem Industry, Bombay. All other chemicals such as buffer components were of the analytical reagent grade and were obtained from British Drug House, Bombay.

Plant material

Ragi (*Eleusine coracana*) seeds var. Purna were obtained from the University of Agricultural Sciences, Bangalore. The seedlings were grown in the Institute nursery for a period of 60 days. Young and fully expanded leaves were used 45 to 60 days after germination. The leaf samples were collected randomly, pooled and divided into several sets for further treatments.

Induction of stress

Sets of leaves weighing 10 g each were taken. The control set of leaves was partly dipped in water to maintain them turgid. The water stress was created by partly dipping the cut end of leaves in 40% polyethylene glycol and exposing the leaves to two 100 W bulbs. The period of stress varied from 2 to 24 h. The turgid tissue was also similarly treated for an identical period except that polyethylene glycol was replaced by water. At the end of each treatment the leaves were washed free of polyethylene glycol, blot-dried, cut into small pieces and chilled. The control set of leaves was also processed similarly. A total of four replications in duplicates were used for each treatment both in control and stress conditions.

Preparation of the homogenate for measuring the activities of ornithine aminotransferase and pyrroline-5-carboxylate reductase

The leaves were ground in a cold pestle and mortar with glass powder (1:1 wt/wt) and 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM pyridoxal-5-phosphate; 1 mM EDTA; and 10 mM β-mercaptoethanol. The resulting slurry was filtered through cheese cloth yielding about 20 ml of liquid. The filtrate was then centrifuged for 10 min at 12,000 *g* in a Sorval RC-5B centrifuge. The supernatant was collected and used for enzyme assays.

Preparation of the homogenate for measuring the activities of proline oxidase and pyrroline-5-carboxylate dehydrogenase

The leaf tissue was ground in a cold pestle and mortar with glass powder (1:1 wt/wt) and 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5% (v/v) triton X-100. The resulting slurry was filtered through cheese cloth and then centrifuged at 1000 *g* for 10 min. The supernatant was used for enzyme assays.

Protein estimation

The soluble proteins present in the leaf extract were precipitated with 10% trichoroacetic acid and the pellet dissolved in O.1N NaOH. The proteins were estimated by the method of Lowry *et al.* (1951).

Free proline estimation

Free proline was estimated by the method of Bates *et al.* (1973).

Preparation of pyrroline-5-carboxylate and o-aminobenzaldehyde

Pyrroline-5-carboxylate was prepared by enzymatic oxidation of proline using rat liver mitochondrial fraction (Johnson and Strecker, 1962). *o*-Aminobenzaldehyde was prepared by chemical reduction of *o*-nitrobenzaldehyde (Smith and Opie, 1955).

Enzyme assays

Ornithine aminotransferase: The assay mixture consisted of 0.1 M potassium phosphate buffer (pH 8.0); 50 mM ornithine; 20 mM α-ketoglutarate; 1 mM pyridoxal-5′-phosphate and enzyme in a total volume of 1.0 ml. After incubation at 37°C, the reaction was terminated by adding 1.0 ml of 10% trichlorocacetic acid. The colour was developed by adding 2 ml of 0.5% *o*-aminobenzaldehyde in 95% ethanol. The mixture was further incubated for 10 min and then centrifuged to remove the denatured proteins. The control tube was devoid of the enzyme extract. The product formed was determined by measuring the absorbance at 440 nm in a Pye-Unicam spectrophotometer. Enzyme activity units were calculated by using a molar extinction coefficient of $2.71 \times 10^3 M^{-1} cm^{-1}$ for pyrroline-5-carboxylic acid.

Proline oxidase

The assay mixture consisted of 15 mM L-proline; 0.01 mM cytochrome c, 0.1 M potassium phosphate buffer (pH 8.0); 0.5% (v/v) triton X-100; and enzyme extract in a total volume of 1.0 ml. The incubation was carried out at 37°C for 30 min and the reaction terminated by adding 1.0 ml of 10% trichloroacetic acid. The colour was developed by incubating the reaction mixture with 2.0 ml of 0.5% *o*-aminobenzaldehyde in 95% ethanol for 10 min. The denatured proteins were removed by centrifugation and absorbance measured against a control at 440 nm. The enzyme extract was not added in the control. Enzyme activity units were calculated as described for ornithine aminotransferase.

Pyrroline-5-carboxylate dehydrogenase

The enzyme activity was monitored by following the increase in absorbance of NADH formed at 340 nm. The reaction mixture consisted of 1 mM pyrroline-5-carboxylate; 0.6 mM NAD^+; 0.1 M potassium phosphate buffer (pH 8.0); 0.5% (v/v) triton X-100 and enzyme extract in a total volume of 3.0 ml. The incubation was carried out at room temperature (20-25°C). The enzyme activity was expressed as nmol of NADH formed/min/g fresh weight.

Pyrroline-5-carboxylate reductase

The enzyme activity was followed by measuring the decrease in absorbance at 340 nm due to oxidation of NADH. The reaction mixture consisted of 1 mM pyrroline-5-carboxylate; 0.12 mM NADH; 0.1 M potassium phosphate buffer (pH 7.4) and enzyme extract in a total volume of 3 ml. The reaction mixture was incubated at room temperature and the decrease in absorbance at 340 nm was followed. The enzyme activity was expressed as nmol of NADH oxidized/min/g fresh weight.

Results

Protein content of the normal and polyethylene glycol treated leaves

The protein content of the normal leaves which were just dipped in water increased from 17.38 ±0.65 mg/g fresh weight at 2 h to 21.79 ±0.23 mg/g fresh weight at 24 h. The protein content of leaves treated with polyethylene glycol showed an increase over the control, which was detectable as early as 2 h after treatment. The increase was as much as 35% over the normal leaves, when the period of treatment was 8 h (P=0.0001). Interestingly enough, soluble proteins were always greater in the stressed leaves. However, the values reached to those of the control on continuing the stress for 24 h (table 1).

Table 1. Changes in the protein content of normal and stressed Ragi leaves.

Period of PEG[a] treatment (h)	mg/g fresh weight	
	Normal leaves*	Stressed leaves*
2	17.38±0.65	19.38±0.87
4	18.61±0.21	20.30±0.30
6	18.61±0.63	22.61±0.65
8	19.95±0.86	27.22±0.52
10	20.38±0.21	27.22±0.75
12	20.76±0.43	25.99±0.21
24	21.79±0.23	22.69±0.43

* Mean of four replications in duplicates ± standard deviation.

[a] Polyethylene glycol.

Increase in free proline content of the leaves subjected to water stress

The free proline levels of the normal leaves varied between 71 μg/g fresh weight to 87 μg/g fresh weight. A very large increase in free proline level was observed in the leaves subjected to water stress. The stressed leaves had 6.5 fold more proline as early as 2 h after polyethylene glycol treatment. The accumulation of proline increased as the stress prolonged and attained maximal levels at 8 h after the polyethylene glycol treatment. The levels remained constant thereafter upto 12 h of the treatment. There was a marginal decrease in the accumulated proline 24 h after the treatment. However, the increase in proline was as much as 65 fold, even on continuing the treatment for 24 h (table 2).

Table 2. Free proline content of normal and stressed Ragi leaves

Period of PEG[a] treatment (h)	μg/g fresh weight	
	Normal leaves*	Stressed leaves*
2	71.39±1.56	465.42±21.63
4	72.48±3.70	1109.39±15.70
6	74.71±2.64	4020.40±14.87
8	82.10±4.36	6939.90±31.88
10	80.80±0.60	6427.08±14.41
12	84.53±1.76	6322.79±14.87
24	87.23±2.73	5969.13±6.93

* Mean of four replications in duplicates ± standard deviation.

[a] Polyethylene glycol.

Ornithine aminotransferase

The activity of ornithine aminotransferase varied from 78 to 133 units at various stages. The enzyme activity increased in leaves treated with polyethylene glycol. The increase was detectable 2 h after the treatment. The enzyme activity was two times larger than in the normal tissue when the treatment was continued for 4 h. The increase reached a maximum value of about 3.5 fold at 8 h of the treatment. Although the enzyme activity declined after 8 h, it was always two fold greater than the respective control values (table 3).

Pyrroline-5-carboxylate reductase

The enzyme activity in the normal leaves varied between 62 to 87 units. The polyethylene glycol treated leaves exhibited an increase in pyrroline-5-carboxylate reductase activity as compared to the activity in the normal leaves. The increase in enzyme activity was proportional to the degree of water stress till 8 h of the treatment, when a maximum increase of about 2.5 fold was observed. An increase of about 1.5 fold was seen after 24 h of the treatment. The pattern of change in enzyme activity was similar to that obtained for ornithine aminotransferase (table 4).

Table 3. Ornithine aminotransferase activity in normal and stressed Ragi leaves.

Period of PEG[a] treatment (h)	nmol of P-5-C[b] formed/min/g fresh weight	
	Normal leaves*	Stressed leaves*
2	77.77± 5.23	144.44± 5.22
4	94.44±13.09	177.78±10.47
6	103.70±10.40	289.43± 0.77
8	114.07± 2.61	399.25± 5.31
10	117.03± 5.27	385.18±20.95
12	124.44± 0.00	374.77±15.71
24	132.66± 5.23	268.89±10.50

* Mean of four replications in duplicates ± standard deviation.

[a] polyethylene glycol.

[b] pyrroline-5-carboxylate.

Table 4. Pyrroline-5-carboxylate reductase activity in normal and stressed Ragi leaves.

Period of PEG[a] treatment (h)	nmol of NADH oxidized/min/g fresh weight	
	Normal leaves*	Stressed leaves*
2	62.20±9.77	81.56± 1.95
4	67.73±5.86	106.44± 1.09
6	73.26± 1.95	138.24±10.55
8	74.64±3.91	171.42±11.73
10	81.56±1.05	145.15±17.59
12	85.70±3.29	131.33± 9.77
24	87.24±7.82	127.18± 3.91

* Mean of four replications of duplicates ± standard deviations.

[a] Polyethylene glycol.

Proline oxidase

The activity of proline oxidase in normal leaves varied from 40 to 44 units at various stages. There was a remarkable decrease in the enzyme activity in stressed leaves. The stress resulted in 50% decrease of enzyme activity at 4 h of the treatment. The enzyme activity was barely detectable after 8 h of treatment. The decrease in the activity was as much as 60 to 80 fold at later stages of water stress (table 5).

Pyrroline-5-carboxylate dehydrogenase

The enzyme activity in the normal leaves ranged between 61 to 75 units. The pattern of change in pyrroline-5-carboxylate dehydrogenase activity was similar to that followed by proline oxidase, though the magnitude of change was not as large. The enzyme activity decreased by about two fold at 6 h of the treatment. The decrease was more pronounced at advanced stages of the treatment. The decrease was as marked as 15 fold in leaves treated with polyethylene glycol for 12 h (table 6).

Table 5. Proline oxidase activity in normal and stressed Ragi leaves.

Period of PEG[a] treatment (h)	nmol of P-5-C[b] formed/min/g fresh weight	
	Normal leaves*	Stressed leaves*
2	40.74±1.04	34.44±1.56
4	41.11±0.25	22.22±2.09
6	42.22±0.53	1.03±0.21
8	44.81±0.70	0.59±0.00
10	44.94±0.35	0.59±0.00
12	42.51±0.23	0.74±0.00
24	44.04±4.70	0.88±0.00

* Mean of four replications in duplicates ± standard deviation.

[a] polyethylene glycol.

[b] pyrroline-5-carboxylate.

Table 6. Pyrroline-5-carboxylate dehydrogenase activity in normal and stressed Ragi leaves.

Period of PEG[a] treatment (h)	nmol of NADH formed/min/g fresh weight	
	Normal leaves*	Stressed leaves*
2	60.95±4.10	47.61±3.83
4	63.86±2.73	37.73±1.36
6	67.73±2.74	32.86±2.73
8	68.70±6.83	13.54±2.73
10	71.99±2.18	7.74±0.20
12	74.51±1.37	5.80±0.74
24	72.57±0.83	21.28±5.58

* Mean of four replications in duplicates ± standard deviation.

[a] polyethylene glycol.

Discussion

A wide array of events occur when water is withheld from plants (Hsio, 1973). Several methods which range from withdrawal of water to plants to the use of chemicals such as polyethylene glycol, mannitol etc., have been employed to create water stress in plants. It has been reasonably well established that polyethylene glycol induced water stress mimics that caused by withdrawal of water from plants (Todd, 1972).

It is evident from the results presented in this paper that the metabolism of Ragi plants responds to the changes in the availability of water. The change in the soluble proteins of the normal leaves over a period of 24 h reflects continuous metabolism occurring in the leaves maintained in turgid conditions. A similar pattern of changes in soluble proteins was observed by Singh *et al.* (1973).

Increased proteolysis and inhibition of protein synthesis have been reported in plants during water stress (Kemble and Macpherson, 1954; Petrie and Wood, 1962). While in most instances a decrease in protein content is noticed during water stress (Vaadia *et al.*, 1961; Shah and Loomis, 1965; Stutte and Todd, 1969), fluctuations in protein content have also been recorded (Chen *et al.*, 1964). The increase in protein levels of leaves subjected to water stress, observed by us could be due to (a) increased *de novo* synthesis and (b) decreased protein degradation or turnover. These alternatives need to be further probed into. In addition to a functional role, the increase in proteins, presumably, helps preserve the leaf structure during the process of wilting. This may represent a survival mechanism for Ragi, a crop grown under rain-fed conditions, has evolved to withstand extreme conditions of water stress.

It is clear from table 2 that proline levels are very high at every period of water stress, stretching over 24 h. Our results depicting proline accumulation are in agreement with other reports (Barnett and Naylor, 1966; Kemble and Macpherson, 1954; Routley, 1966; Singh *et al.*, 1973). The greater accumulation of proline in these genotypes, presumably, render them drought tolerant. This hypothesis is being tested at the present time. An increase in soluble proteins during water stress and the dramtic increase in the proline levels compared to other amino acids in the free amino acid pool clearly suggest that the contribution of proteolysis may not be the major factor responsible for this phenomenon. A possible reason for these increased levels of proline during water stress could be an alteration in the activities of the enzymes involved in the biosynthesis and degradation of proline. The pathway for the biosynthesis and degradation of proline as established in micro-organisms is shown in figure 1.

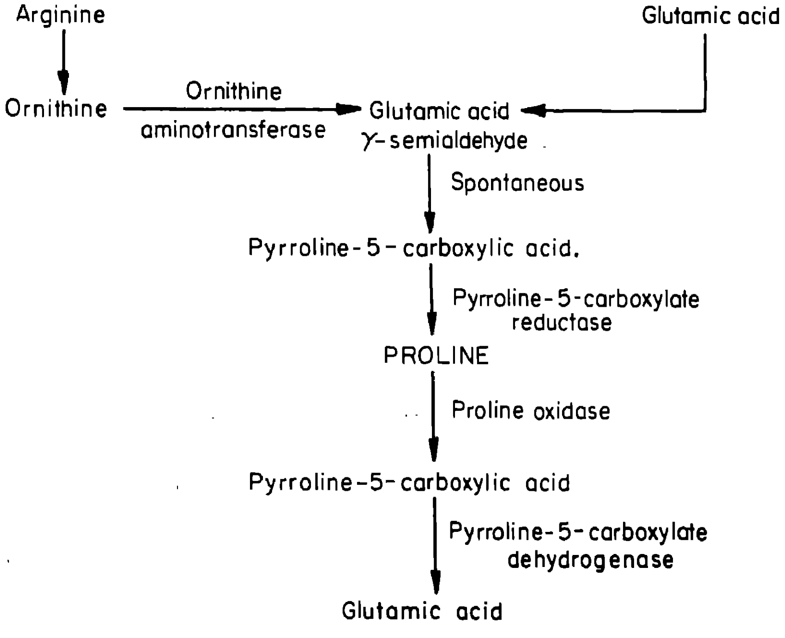

Figure 1. Pathway for proline metabolism.

The detection of various enzymes of pathway metabolism for the first time in any plant system, clearly suggests the operation of this pathway in plants. The results presented in this study show that the activities of ornithine aminotransferase and pyrroline-5-carboxylate reductase increase by about 3.5 and 2.5 fold respectively suggesting that enhanced synthesis of proline may be occuring due to increased activities of these enzymes during water stress. Concomittantly, the activities of enzymes responsible for the degradation of proline *viz.*, proline oxidase and pyrroline-5-carboxylate dehydrogenase decrease dramatically. These two factors together, probably, result in the increased levels of proline. Our results at the present time cannot distinguish between the two alternatives responsible for this phenomenon viz., (i) structural modification of the existing enzymes and the loss of regulatory restraints or (ii) altered rates of synthesis and degradation of the enzyme proteins. Our preliminary observations (unpublished) show that the activity of ornithine aminotransferase is altered during water stress due to a change in the enzyme protein as indicated by an alteration in its temperature and pH activity profiles and as well as a change in substrates (ornithine and α-keto glutarate) saturation patterns of the partially purified enzyme isolated from Ragi leaves subjected to water stress. These observations provide some support to the first alternative mentioned above and also indicate that the change in the activities may not be due to the presence of an inhibitor/activator in water stressed Ragi leaves. We are at present investigating the causes for the changes in the activities of the other enzymes of proline metabolism and until these studies are completed, the presence of a naturally occurring inhibitor/activator as a consequence of water stress can not be ruled out.

These results delineate the pathway for the biosynthesis and degradation of proline, emphasize the role of proline in the metabolism of plants subjected to water stress and suggest possible reasons for the increased levels of proline observed during water deprivation.

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Enzyme inhibitors from plants: Enterokinase inhibitors in tubers and seeds

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Abstract. Of the 22 tubers and 9 pulses screened for inhibitors of enterokinase activity, the following 12 tubers, *Curcuma amada, Kyllinga monocephala, Solanum tuberosum, Canna indica, Helianthus tuberosus, Coleus parviformis, Mirabilis jalapa, Colocasia antiquorum* (red variety), *Alium cepa, Amorphophalus companulatus, Maranta arundinacea, Daucus carota,* and 9 pulses namely, *Vigna sinensis, Arachis hypogea, Pisum sativum, Phaseolus vulgaris* (white bean), *Phaseolus vulgaris* (kidney bean), *Phaseolus mungo, Cicer arietinum, Dolichos lablab* and *Cajanus cajan* contained inhibitory activity. Three tubers, *Amorphophalus companulatus, Maranta arundinacea* and *Daucus carota* and all the nine pulses exhibited endogenous esterase activity towards benzoyl arginine ethyl ester. Among the 8 pulses and 3 tubers processed by affinity chromatography on trypsin-sepharose, to separate trypsin inhibitor from enterokinase inhibitor, *Phaseolus vulgaris* (kidney bean), *Phaseolus vulgaris* (white bean) and *Dolichos lablab* contained distinct enterokinase inhibitors. These fractions were devoid of trypsin inhibitor activity. The trypsin inhibitor from *Coleus parviformis* tubers alone did not bind to trypsinsepharose and was recovered in the unbound fraction along with the enterokinase inhibitor.

Keywords. Enterokinase inhibitors; trypsin inhibitors; proteinase inhibitors; tubers; pulses.

Introduction

Enterokinase (EC 3.4.21.9) is a serine proteinase required for the activation of pancreatic proenzymes in the duodenum. Porcine enterokinase specifically cleaves a peptide bond lys 6-ile 7 of bovine trypsinogen (Blackburn, 1976) to convert the proenzyme to active trypsin and initiates the cascade of reactions leading to the activation of many digestive proteinases (Blackburn, 1976). The occurrence of enterokinase deficiency in human infants and the consequent protein malabsorbtion in such individuals have provided *in vivo* evidence for the pivotal role of the enzyme in the digestion process (Hadorn *et al.*, 1969; Howarth *et al.*, 1975). Any factor in food which is capable of suppressing the enterokinase activity would lead to disturbances in digestion comparable to enterokinase deficiency. In view of this, a systematic survey of edible plant tissues for

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Abbreviations used: N-B_z-L-arg, α-N-benzoyl-L-arginine; N-B_z-DL-arg, α-N-benzoyl-DL-arginine.

enterokinase inhibitor is of interest. Although inhibitors of pancreatic proteinases like trypsin and chymotrypsin have been extensively investigated, the presence of enterokinase inhibitors in food material was reported only recently (Lau *et al.*, 1980).

In this paper, we report the occurrence of enterokinase inhibitors in a wide variety of plants, and also demonstrate for the first time the presence of a specific enterokinase inhibitor, free of trypsin inhibitory activity in some plant seeds.

Materials and methods

Materials

The tubers and seeds were procured locally from commercial sources. A few wild varieties of non-edible tubers were collected during the rainy season.

α-N-Benzoyl L-arginine (N-B_Z-L-arg)ethyl ester, α-N-benzoyl DL-arginine(N-B_Z-DL-arg) *p*-nitroanilide and Trizma base (tris hydroxymethyl amino methane) were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Cyanogen bromide activated-Sepharose 6MB and Sephadex G-15 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine trypsin (EC 3.4.21.4, twice crystallized) was the product of Worthington Biochemical Corporation, Freehold, New Jersey, USA.

Bovine trypsinogen was prepared as described by Wilimowska-Pelc and Mejbaum-Katzenellenbogen (1978) taking care to obtain the trypsinogen free of trypsin contamination. Bovine enterokinase was partially purified from duodenum according to Liepnicks and Light (1979). The procedure was followed upto the ammonium sulphate fraction stage and this fraction was used as the source of bovine enterokinase in all the studies. Trypsin-Sepharose was prepared as described earlier (Shivaraj and Pattabiraman, 1981).

Methods

Preparation of cell-free extracts: Tubers were homogenized in a glass mortar with 3 volumes of 0.1 M Tris-HCl buffer, pH 8.0 at 28-30°C. The homogenate was centrifuged at 10,000 *g* for 20 min, the supernatant was separated and used to estimate enterokinase inhibitory activity and endogenous esterase activity. Seeds were extracted with 10 volumes of the same buffer as described above.

Assay of enterokinase: Esterase activity of bovine enterokinase was determined using N-B_Z-L-arg ethyl ester as substrate by the method described by Bhat *et al.* (1978) for trypsin. The assay system contained 60 μmol of Tris-HCl buffer pH 8.0; 1 μmol of $CaCl_2$; 2 μmol of N-B_Z-L-arg ethyl ester and suitable aliquots of enterokinase preparation in a final volume of 1.0 ml. The reaction was initiated by the addition of the substrate. The other details of assay were similar to those described elsewhere (Bhat *et al.*, 1978). The crude extracts of different plant tissues (0.1-0.6 ml) were included in the assay system to determine the inhibitory activity. The difference between the esterase activity of enterokinase control and test containing the enterokinase plus the crude extracts was the measure of inhibitory activity. At the concentrations used, the substrate was not a limiting factor. To estimate esterase activity of the plant extract, the assay system was modified by including the crude extract in a reaction mixture containing N-B$_Z$-L-arg ethyl ester, $CaCl_2$ and buffer as described above but omitting enterokinase. One unit of esterolytic activity is defined as the amount of enzyme which liberates 1 µmol of benzoyl arginine from N-B$_Z$-L-arg ethyl ester in 1 min under the assay conditions. One unit of esterolytic inhibitor is the amount which suppresses the esterolytic activity by one unit.

Assay of enterokinase activity using trypsinogen as substrate was performed based on the following reactions.

$$\begin{tikzcd}
\text{Trypsinogen} \arrow[rr, "\text{enterokinase}"] & & \text{trypsin} \arrow[d] \\
\text{N-B}_Z\text{-DL-arg-p-nitroanilide} \arrow[rr] & & \text{benzoyl arginine} + \text{p-nitroaniline}
\end{tikzcd}$$

Trypsinogen was activated to trypsin by preincubation with enterokinase at pH 6.0 and trypsin formed was assayed by the method of Erlanger *et al.* (1961).

The preincubation system contained 50 µmol Tris acetate buffer pH 6.0; 1 µmol, $CaCl_2$; 100 µg trypsinogen; and suitable amounts of enterokinase preparation in a final volume of 1.0 ml. The reaction was initiated by adding trypsinogen and incubated at 37°C for 10 min. To this assay mixture, 2.0 ml of 2 mM N-B$_Z$-DL-arg p-nitroanilide in 50 mM Tris-HCl, buffer, pH 8.0 was added and incubated for further period of 15 min. The reaction was stopped by adding 1.0 ml of 30% acetic acid. The colour was measured at 410 nm.

One unit of enterokinase estimated is defined as that amount which produced one amidolytic unit of trypsin under the specified assay conditions. One amidolytic unit of trypsin is that amount which liberated 1 µmol of p-nitroaniline in 1 min. Enterokinase inhibitor activity was estimated by this method by including suitable aliquots of fractions in the preincubation systems. One unit of enterokinase inhibitor is the amount which suppressed the enterokinase activity by one unit.

Amidolytic activity of bovine crystalline trypsin was estimated as follows. One µmol of $CaCl_2$, 100 µmol Tris-HCl buffer pH 8.0; 4 µmol N-B$_Z$-DL-arg p-nitroanilide and 20 µg of trypsin in a final volume of 3.0 ml were incubated at 37°C for 15 min. The reaction was stopped by the addition of 1.0 ml of 30% acetic acid and the absorption measured at 410 nm. To estimate trypsin inhibitor activity, the aliquots of crude extracts and chromatographic fractions were included in the assay system and the amidolytic activity was determined. The difference between the amidolytic activity of control and test determined in the absence and presence of plant extracts was a measure of the inhibitor activity. One amidolytic unit of trypsin is that amount which hydrolyzed 1 µmol of N-B$_Z$-DL-arg p-nitroanilide in 1 min. One unit of trypsin inhibitor is that amount which suppressed the activity of trypsin by one unit. Protein was estimated by the method of Lowry *et al.* (1951).

Affinity chromatography on trypsin-sepharose: To explore the possibility of the presence of a specific enterokinase inhibitor devoid of trypsin inhibitor activity, crude extracts were subjected to affinity chromatography on trypsin-Sepharose at 4°C. The crude extract (2.7 ml) was mixed with 0.3 ml of 5 M NaCl and applied to a 5.5 ml (0.9×8.6 cm) column of trypsin-sepharose previously equilibrated with 10 mM Tris-HCl buffer pH 8.0 containing 0.5 M NaCl. The flow rate was 10 ml/h. The column was washed with 50 ml of the equilibration buffer and then eluted with 50 ml of 50 mM HCl. Ten ml fraction were collected. Each fraction was dialyzed overnight against 1 litre of 10 mM Tris-a-acetate buffer, pH 6.0 at 4°C. Suitable aliquots (5 µl-300 µl) were used for measuring enterokinase and trypsin inhibitor activities.

Results

Twentytwo edible as well as non-edible tubers and 9 pulses were screened for enterokinase inhibitory activity with N-B_Z-L-arg ethyl ester as substrate. The results are presented in table 1. The plants were classified into two groups based on the presence of enterokinase inhibitor and endogenous esterase activity.

Table 1. Enterokinase inhibitor and endogenous esterase activities of tubers and seeds.

	Endogenous esterase		Enterokinase inhibitor	
	Specific activity (units/g protein)	Units[a]/100 g tissue	Specific activity	Units[a]/100 g tissue
A				
Mango ginger (*Curcuma amada*)	Nil	Nil	47.1	8.9
Kylinga (*Kyllinga monocephala*)	,,	,,	28.3	18.9
Potato (*Solanum tuberosum*)	,,	,,	21.5	18.9
Canna (*Canna indica*)	,,	,,	21.3	12.3
Dahlia (*Helianthus tuberosus*)	,,	,,	20.8	12.3
Coleus (*Coleus parviformis*)	,,	,,	13.2	17.9
Mirabilis (*Mirabilis jalapa*)	,,	,,	10.7	15.4
Colocasia (*Colocasia antiquorum*, red variety)	,,	,,	8.2	7.9
Onion (*Allium cepa*)	,,	,,	5.2	3.5
B				
Amorphophalus (*Amorphophalus companulatus*)	98.2	229.8	21.5	50.4
Arrowroot (*Maranta arundinacea*)	2.9	4.9	15.6	26.6
Carrot (*Daucus carota*)	24.6	14.0	12.3	7.0
Cow pea (*Vigna sinensis*)	6.6	112.5	5.2	87.8
Pea nut (*Arachis hypogea*)	0.9	20.7	3.8	83.7
Green pea (*Pisum sativum*)	4.4	72.0	3.4	56.0
White bean (*Phaseolus vulgaris*)	5.2	77.0	3.3	49.0
Kidney bean (*Phaseolus vulgaris*)	9.7	105.0	3.1	34.0
Black gram (*Phaseolus mungo*)	6.0	91.0	2.8	42.0
Bengal gram (*Cicer arietinum*)	9.9	77.0	2.7	21.0
Field bean (*Dolichos lablab*)	3.1	37.5	2.5	30.0
Red gram (*Cajanus cajan*)	2.5	38.3	1.3	20.3

[a] Enterokinase esterase activity is assessed using N-B_Z-L-arg ethyl ester as substrate.

The values are the mean of three different estimations.

Group A includes nine tubers which exhibit only enterokinase inhibitor activity. Three tubers and 9 pulses showed both enterokinase inhibitor activity and endogenous esterase activity and are included in group B. Other plant materials namely tubers of *Diascorea alata, Curcuma longa, Musa paradisica, Colocasia antiquorum* (garden variety), *Elettaria cardamommum* were devoid of inhibitor activity as well as endogenous esterase activity while *Diascorea esculenta, Amorphophalus companulatus, Arginia indica, Alocasia macrohiza* and *Ipomea batata* exhibited only endogenous esterase activity.

Among the tubers, *Curcuma amada* exhibited the highest specific activity of enterokinase inhibitor (47.1×10^{-3} units/mg protein). The specific activity of the inhibitor was found to be highest in *Vigna sinensis* among pulses. The specific activity of the inhibitor in pulses varied from 1.3 to 5.2×10^{-3} units/mg protein and was lower than that in tubers ($5.2\text{-}47.1 \times 10^{-3}$ units/mg protein). Maximum specific activity of endogenous esterase was observed in *Amorphophalus companulatus*. Endogeneous esterase specific activity varied from 0.9 to 9.9×10^{-3} in different pulses. The activity of enterokinase inhibitor in pulses in terms of units/100 g tissue was higher than in tubers.

An attempt at separation of enterokinase inhibitor from trypsin inhibitor was made by chromatography of the crude extracts on trypsin-sepharose. The data are presented in table 2.

Table 2. Quantitation of enterokinase inhibitor and trypsin inhibitor activities after trypsin-sepharose affinity chromatography.

	Total protein applied (mg)	Enterokinase inhibitor in washings (Units[a]/g)	Trypsin inhibitory activity in bound fraction (Units[b]/g)
Kidney bean (*Phaseolus vulgaris*)	39.0	6.42	11.51
Field bean (*Dolichos lablab*)	43.5	1.04	12.46
White bean (*Phaseolus vulgaris*)	57.0	0.77	5.42
Coleus (*Coleus parviformis*)	20.2	0.25[b]	Nil
Black gram (*Phaseolus mungo*)	58.5	Nil	2.03
Green pea (*Pisum sativum*)	74.0	,,	2.07
Bengal gram (*Cicer arietinum*)	40.0	,,	5.82
Cow pea (*Vigna sinensis*)	39.0	,,	8.33
Pea nut (*Arachis hypogea*)	27.0	,,	1.29
Arrowroot (*Maranta arundinacea*)	23.2	,,	1.52
Potato (*Solanum tuberosum*)	4.5	,,	1.27

[a] Enterokinase inhibitor activity was estimated by trypsinogen activation followed by amidolytic determination of trypsin.

[b] The enterokinase inhibitor activity was the difference between the total inhibitory activity and the activity against trypsin. The trypsin inhibitory activity was 0.20 units/g.

The unbound fractions were found to contain enterokinase inhibitors in the cases of kidney bean, field bean, white bean and coleus tuber. The trypsin inhibitor was found in the bound fraction and was eluted in 50 mM HCl except in the case of coleus where trypsin inhibitor was not bound to trypsin-sepharose at all. In all other cases investigated, no separate enterokinase inhibitor could be detected in the unbound fraction. Due to dilution of the fractions during trypsin-sepharose affinity chromatography, assessment of inhibitor activity against the esterolytic action of enterokinase could not be carried out. Elution pattern of enterokinase inhibitor and trypsin inhibitor on trypsin-sepharose affinity chromatography of extracts of kidney bean, white bean and field bean are shown in figure 1. The early fractions which contained enterokinase inhibitor activity were devoid of trypsin inhibitor activity.

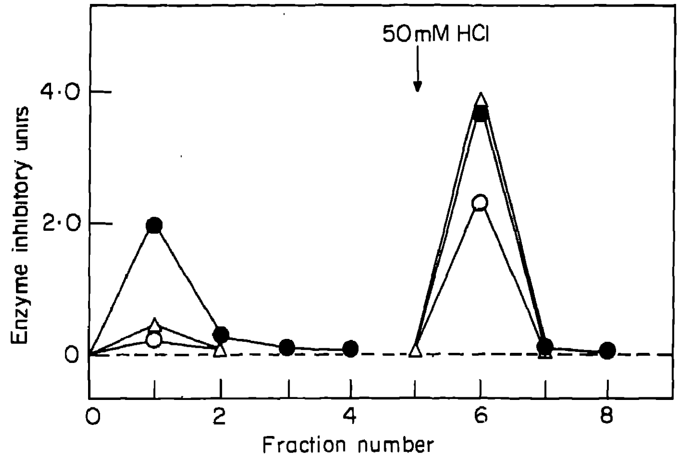

Figure 1. The crude extracts from kidney bean, field bean and white bean were subjected to affinity chromatography on trypsin sepharose as described under methods. O, kidney bean; Δ, field bean; ●, white bean.

The proteinase inhibitors in coleus provided a distinct picture. The trypsin inhibitor and enterokinase inhibitor of coleus could not be separated by this method, as the trypsin inhibitor did not bind to the column.

Discussion

The presence of proteinaceous inhibitors against the digestive proteinases in nature is well documented (Ryan, 1973). Although the similarity of enterokinase to other serine proteases like trypsin and chymotrypsin has been studied, this enzyme was not investigated in detail with respect to its susceptibility to inhibition by various natural proteinase inhibitors. Recently Liepnicks and Light (1979) reported that pancreatic trypsin inhibitor is a potent inhibitor of enterokinase. Lau *et al.* (1980) demonstrated the presence of enterokinase inhibitor in potato tubers, tomato leaves and peanut seeds. The peanut seed enterokinase inhibitor was purified and has been fractionated into two inhibitors which showed trypsin inhibitory and chymotrypsin inhibitory activities, indicating the non-specific nature of the inhibitors (Lau *et al.*, 1980).

The results presented revealed the presence of enterokinase inhibitor in storage tissues of 21 plants. Among these plant materials, 8 pulses and 3 tubers were further investigated by affinity chromatography. Specific enterokinase inhibitors in 3 of them namely, kidney bean, white bean and field bean need to be highlighted as the tissues which possess the enterokinase inhibitor and were devoid of trypsin inhibitor activity. The presence of inhibitor with such specificity towards enterokinase has not been reported so far. The presence of trypsin inhibitors in the plant storage tissues invetigated limits the assessment of enterokinase inhibitory activity with trypsinogen as substrate. In view of this, esterase activity of enterokinase with N-B_zL-arg ethyl ester as substrate was used in the screening studies. The extracts of *Alocasia macrorhiza* and ragi (*Eleusine coracana*) were found to exhibit no inhibitor activity against the esterase activity of our enterokinase preparation. This observation confirmed the assumption that the enterokinase preparation is free of trypsin contamination. If the enterokinase preparation were to be contaminated with trypsin, significant esterase inhibitor activity would have been observed in alocasia (Sumathi and Pattabiraman, 1977) and ragi (Shivaraj and Pattabiraman, 1981) extracts as these two are known to contain potent trypsin inhibitors.

The inhibition of esterase activity of enterokinase by crude extracts may be due to the nonspecific action of trypsin inhibitors in the case where separation of enterokinase inhibitor was not possible by affinity chromatography. Coleus tuber presented a different type of trypsin inhibitor which did not bind to trypsin-sepharose. This may be due to very mild interaction of the inhibitor with trypsin or due to the cleavage of the inhibitor during interaction and its release. The present report adds many pulses and tubers to the list of sources of enterokinase inhibitors. Further studies to isolate and characterize enterokinase inhibitor from pulses are in progress.

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Studies on the uptake of benzanthrone by rat skin and its efflux through serum

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Abstract. The uptake of benzanthrone by rat skin showed saturation kinetics and was dependent upon the weight of skin and time, temperature and pH of the incubation medium. Heating of segments above 50°C caused significant lowering of the uptake. The uptake was irreversibly inhibited by $HgCl_2$ and not by sodium arsenate, KCN, NaF, *p*-chloromercuribenzoate, N-ethyl-maleimide, cycloheximide, iodoacetic acid and 2,4-dinitrophenol suggesting that the uptake was not energy-dependent. Lipid micelles of the skin accounted for a part of the binding. Most of the benzanthrone taken up by the skin was effluxed through serum proteins.

Keywords. Benzanthrone; dye intermediate; uptake by the skin; serum transport.

Introduction

Benzanthrone |7H-benz(de)anthracen-7-one|, an anthraquinone derivative, is a dye intermediate commonly used for the synthesis of a number of vat and disperse dyes. Workers exposed to benzanthrone during its manufacture, pulverization and storage develop itching, burning sensation, erythema and pigmentation of the exposed as well as covered parts of the skin (Singh *et al.*, 1967; Trivedi and Niyogi, 1968; Singh and Zaidi, 1969; Singh, 1970). In acute stages, the skin becomes tender, rough and peals off easily. Singh *et al.* (1967) showed that prolonged exposure to benzanthrone in experimental animals stimulates cell growth of all the skin layers together with increased vascularity and fibrosis. These histopathological reports indicate that the skin is equipped with a mechanism for the uptake of benzanthrone. In the present investigation, an attempt has been made to study the *in vitro* kinetics of benzanthrone uptake by skin and its transport into the serum.

Materials and methods

Chemicals

Benzanthrone, obtained from Indian Dyestuff Industries, Kalyan, was purified on a neutral alumina column using 1,2-dichloroethane as an eluant. The pooled active

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Abbreviation used: KRB, Krebs-Ringer bicarbonate buffer.

fractions were concentrated in a Buchi Rotavapor and finally dried in a vacuum oven. It gave one spot (Rf value, 0.58) by thin layer chromatography on silica gel-G plates using toluene as solvent system (Swicki *et al.,* 1966). The purity of benzanthrone was further assessed according to the Indian Standard specifications (IS: 5044-1969), infra-red (IR), nuclear magnetic resonance (NMR) and Mass spectroscopy. Iodoacetic acid and N-ethylamaleimide were products of Sigma Chemical Co., St. Louis, Missouri, USA. *p*-Chloromercuribenzoate and glutathione were procured from V.P. Chest Institute, New Delhi. 2,4-Dinitrophenol and 2-mercaptoethanol were the respective products of B.D.H., Poole, England and E. Merck, Dermstadt, Germany. All other chemicals used were of the Analytical Reagent grade.

Preparation of skin segments

Male albino rats, weighing approximately 200 g, were drawn from Indian Toxicology Research Centre's stock colony. The dorsal surface of the skin was shaved with an Oster electric clipper (model A5-00) and the animals sacrified by decapitation. Skin pieces from the shaved area were cut and soaked in chilled 150 mM KCl solution as described in the procedure of Sahib and Krishna Murti (1969).

Uptake studies

To each 25 ml Erlenmeyer flasks containing 0.3-0.4 g portions of skin segments and 5.0 ml of Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, 200 µg of benzanthrone (from 1 mg/ml ethanolic stock) was added. The blanks contained equal volume of ethanol. The flasks covered with aluminium foils were shaken in a metabolic shaker (120 strokes/min) for the required period. The effects of increasing weight of skin segments (0.1-0.5 g), varying concentrations of benzanthrone (50-500 µg) and time (10-180 min); temperature (25-70°C); inhibitors, namely, sodium arsenate, KCN, NaF, $HgCl_2$, *p*-chloromercuribenzoate, N-ethylmaleimide, cycloheximide, iodoacetic acid and 2,4-dinitrophenol (at 0.01, 0.1 and 1.0 mM levels); reducing agents, namely, ascorbic acid, cysteine, sodium dithionite and sodium metabisulphite all at 1.0 mM concentration and pH 5.0-10.0 of the incubation medium, on the uptake of benzanthrone were studied. After incubation, the skin segments were washed, homogenized and used for the determination of benzanthrone. Alternatively, the washings were mixed with the supernatant and benzanthrone in the supernatant was extracted with 1,2-dichloroethane and estimated. The reliability of the extraction procedure was confirmed by recovery experiments in which known amounts of benzanthrone were added to the KRB-buffer and extracted with 1,2-dichloroethane in exactly the same manner as that of experimental animals. The recovery was between 98-100%.

Efflux studies

Skin segments preincubated with 200 µg benzanthrone were washed twice with chilled KRB-buffer, pH 7.4 and transfered to flasks containing 5.0 ml of rat serum and shaken for 2 h. The skin segments were then removed and homogenized in 1,2-dichloroethane for the estimation of the remaining bound benzanthrone. Its transport into serum was calculated. The effect of serum dilutions (0.0, 2.5, 5.0, 7.5 and 10.0 fold) and contact period (15-180 min) on the transport of benzanthrone was studied.

Preparation of homogenate

Skin segments were thrown into liquid air, the frozen pellets thawed at room temperature and the operation repeated to get a pulpy mass that could be homogenized in a power-driven Potter-Elvehjem homogenizer with teflon pestle. The homogenate was centrifuged at 2000 *g* for 15 min in a Remi T8 centrifuge. The homogenate was prepared in 1,2-dichloroethane to extract the bound benzanthrone from the skin segments completely.

Preparation of lipid micelles

Lipids were extracted by homogenizing the skin segments with a chloroform-methanol (2:1) mixture according to the procedure of Folch *et al.* (1957). The lipids thus extracted were dispersed in 50 mM phosphate buffer pH 7.5, and exposed to ultrasonic waves in a Vibronics Ultrasonic Processor (model VPL-P2) for 10 min at 20 KHz and 150 mA current. After sonic disruption samples were centrifuged at 5°C at 1,00,000 *g* for 60 min in an ultracentrifuge (MSE superspeed 75). The supernatant was used as the source of lipid micelles (Kapoor *et al.*, 1972).

Binding of benzanthrone to lipid micelles

Skin lipid micelles (1-10 mg) were allowed to interact with 50 µg of benzanthrone in 50 mM phosphate buffer pH 7.5 for 1 h at 37°C. The unbound benzanthrone was recovered by centrifugation at 2000 *g*, in a Remi T8 centrifuge for 15 min extracted with 1,2-dichloroethane and estimated against suitable blanks.

Estimation of benzanthrone

Benzanthrone was estimated by measuring the absorbance at 385 nm in an Unichem SP-500 spectrophotometer. The molar extinction coefficient (ε) of benzanthrone was 1.175×10^4 litre mol^{-1} cm^{-1} at 385 nm in 1,2-dichloroethane.

Results

Uptake studies

Effect of varying concentrations of benzanthrone: The rate of uptake as affected by concentration of benzanthrone in the medium is shown in figure 1. Increase in the concentration of benzanthrone upto 300 µg gives a linear relationship on the uptake per g of skin. Further increase in concentration does not enhance the uptake.

Effect of skin weight: The uptake of benzanthrone by skin from the medium increases linearly on increasing the weight of the skin as illustrated in figure 2.

Effect of incubation time: Figure 3 shows the effect of time of incubation of skin segments in the medium containing benzanthrone. Maximum uptake was observed at 70 min. Further increase in incubation time showed no effect on the total uptake of benzanthrone.

Effect of incubation temperature: The uptake (after 70 min of incubation) is initially temperature-dependent. maximum uptake is observed between 40-45°C, after which further increase in temperature decreases the uptake of benzanthrone (figure 4).

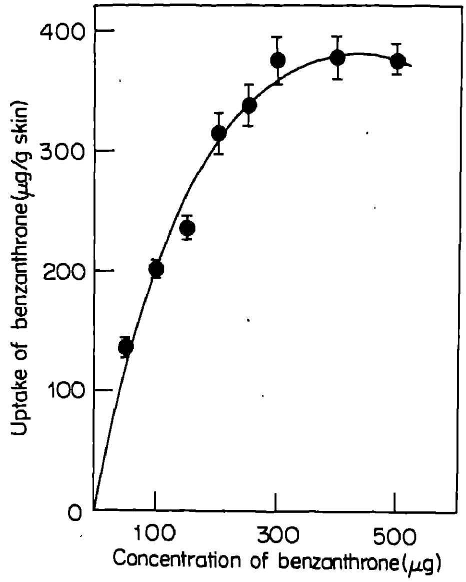

Figure 1. Concentration dependence in relation to benzanthrone uptake.

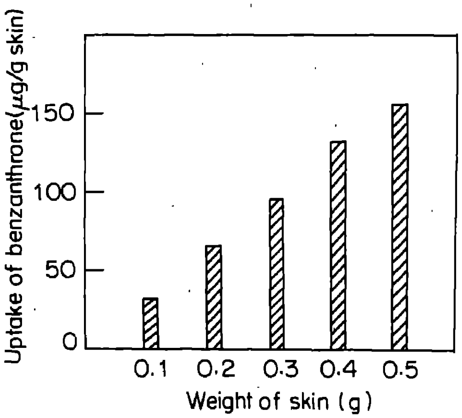

Figure 2. Effect of skin weight on the uptake of benzanthrone

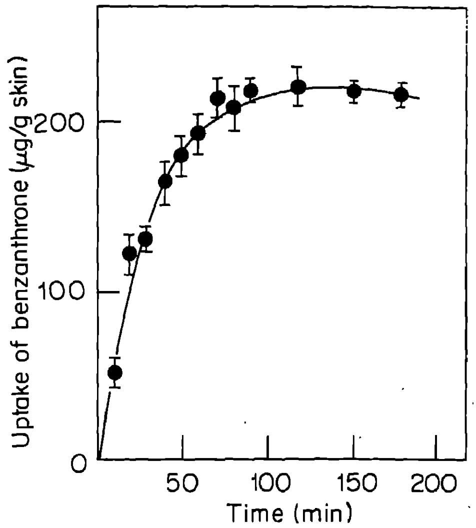

Figure 3. Uptake of benzanthrone in response to exposure time.

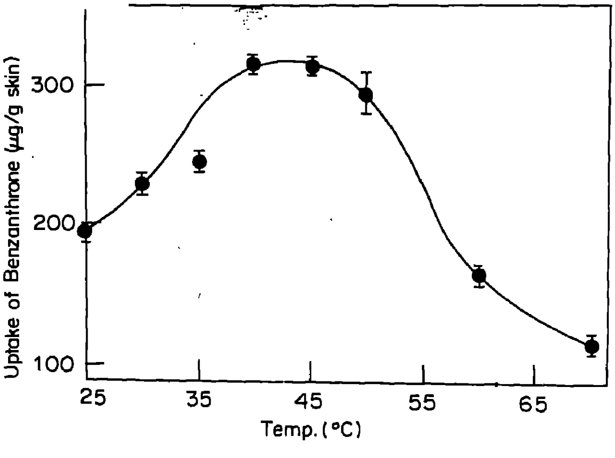

Figure 4. Effect of temperature on the uptake of benzanthrone.

Effect of pH: The uptake of benzanthrone is increased as the pH of the incubation medium is increased from acidic to neutral. The optimum pH range is 7.2-7.6. Further increase towards the alkaline region leads to a slow decrease in the uptake (figure 5).

Effect of inhibitors: Sodium fluoride, iodoacetate, sodium arsenate, KCN, dinitrophenol and cycloheximide do not inhibit the uptake of benzanthrone at

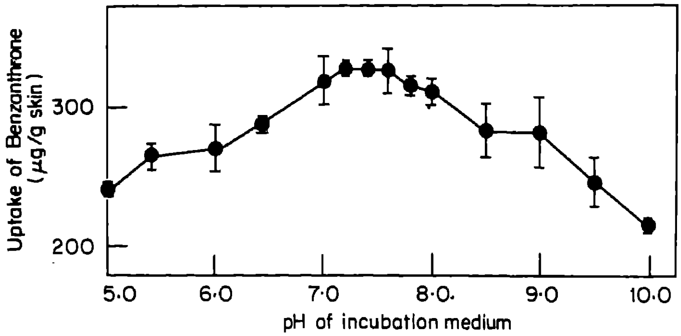

Figure 5. Influence of pH on the uptake of benzanthrone.

0.01, 0.1 and 1.0 mM levels. $HgCl_2$ has no effect at 0.01 and 0.1 mM. An inhibition of 43% in the uptake was observed at the highest concentration tested (1.0 mM) (table 1). The inhibition is not reversed by adding reduced glutathione and 2-mercaptoethanol to a final concentration of 1.0 mM.

Table 1. Effect of inhibitors on the uptake of benzanthrone by rat skin.

	Uptake of benzanthrone (μg/g skin)		
	Concentration of inhibitor (mM)		
	0.01	0.1	1.0
Nil	–	312	–
Sodium arsenate	310	308	303
KCN	316	311	312
NaF	311	309	307
$HgCl_2$	311	294	176
p-Chloromercuribenzoate	318	299	303
N-Ethylmaleimide	305	305	307
Cycloheximide	310	308	308
2,4-Dinitrophenol	308	306	306
Iodoacetic acid	310	303	305

Effect of reducing agents: Ascorbic acid, cysteine, sodium dithionite and sodium metabisulphite have no effect on the uptake at the concentration tested (1.0 mM) (table 2).

Table 2. Effect of reducing agents on the uptake of benzanthrone.

Reducing agent (1.0 mM)	Benzanthrone uptake (μg/g skin)
Control	320
Ascorbic acid	316
Cysteine	318
Sodium dithionite	317
Sodium metabisulphite	324

Transport studies

Effect of serum concentration: Undiluted serum can transport out 84% of the skin-bound benzanthrone (in 120 min of incubation). With 5 and 10 fold dilution, 40 and 13.5% benzanthrone is realised respectively indicating thereby that higher the dilution of serum lesser is its transport (table 3).

Table 3. Relationship between benzanthrone transport and serum concentration.

Fold dilution of serum	Transported benzanthrone	
	(μg/g skin)	(% transport into the serum)
No serum	321	0
0.0	270	84
2.5	199	62
5.0	128	40
7.5	86	27
10.0	43	13.5

Effect of contact time on transport: The transport of benzanthrone into serum gradually increases with contact time. Maximum transport is encountered at 120 min after which further increase in contact time has no influence on the total transport (figure 6).

Binding study with isolated lipid micelles: As illustrated in table 4, binding of benzanthrone is linearly dependent on the amount of skin lipid micelles up to 10.0 mg levels as tested in the present study.

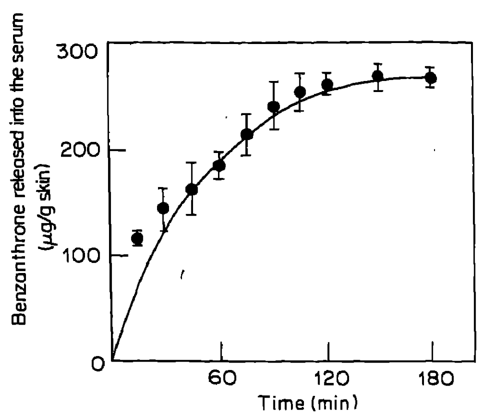

Figure 6. Relationship between benzanthrone transport and contact time.

Table 4. Binding of benzanthrone to rat skin lipid micelles.

Skin lipid micelles (mg)	Benzanthrone bound (μg)
1.0	4.6
2.5	9.2
5.0	18.3
7.5	28.8
10.0	38.3

Discussion

The present study shows that rat skin could pick up benzanthrone from the medium and the skin bound benzanthrone could then be transported through serum.

The uptake is temperature-dependent initially and increases exponentially with rising temperature. This may be largely due to changes in the membrane diffusion constant (Blank *et al.*, 1967) causing a decrease in the activation energies for diffusion across the stratum corneum (Scheuplein and Blank, 1971). The binding is sensitive to temperature above 50°C as the uptake is significantly decreased. Collagen, the major structural protein of skin, is known to undergo thermal denaturation at 52-53°C (Gross 1964). This may suggest that collagen is possibly one of the factors involved in the binding. Separate experiments with reconstituted collagen fibrils and the effect of temperature are in agreement with this finding (Joshi *et al.*, personal communication). Within a pH range of 5.0-10.0, maximum uptake occurs around pH 7.0-7.6. This behaviour is somewhat different from that of albumin, the binding property of which is limited to a narrow pH range (Hargreaves, 1968).

The uptake of benzanthrone is decreased by $HgCl_2$ at 1.0 mM concentration. The inhibition is not likely to be due to the involvement of $-SH$ groups because $-SH$ binding agents such as *p*-chloromercuribenzoate and N-ethylmaleimide had no effect on the uptake; glutathione and 2-mercaptoethanol when added to the final concentration of 1.0 mM were without effect on the uptake. The inhibition by $HgCl_2$ may be either due to the irreversible binding at the same site on the skin at which the benzanthrone binds, or due to its interaction with some other site on the skin not directly involved in the binding of benzanthrone which may result in a conformational change due to which the binding ability is decreased. The uncouplers and inhibitors of oxidative phosphorylation showed no change on the benzanthrone uptake kinetics indicating it was not an energy dependent process. The absence of metabolic processes in the dead keratinizing layers precludes any role of active transport processes (Malkinson and Gehlmann, 1977) and molecules may diffuse across the stratum corneum by purely passive means (Treager,1966).

No work on the metabolism of benzanthrone by skin has been reported this far. However, this possiblity can not be ruled out under *in vivo* conditions. In order to confirm whether metabolism of benzanthrone takes place under our experimental condition and whether such metabolites might also be included in the estimation procedure, we performed a separate experiment with a view to identify the metabolites formed, if any by chromatography. Results of this study indicated the presence of only one spot corresponding to benzanthrone confirming that no metabolism occurs in skin segments.

Serum proteins appear to have an affinity for benzanthrone as the skin-bound benzanthrone was transported into serum. The release of benzanthrone increased with increasing serum protein concentration. Acton (1968) showed that the fraction of a bound chemical increase with increasing protein concentration. A likely competition for benzanthrone between binding sites of the skin and serum proteins may exist. When all the binding sites of serum proteins are saturated there is no more efflux. A separate study (Joshi et al., personal communication) in which the protein bound benzanthrone was fractionated on Sephadex G-200 column, revealed that benzanthrone has more affinity for albumins than the globulin fractions.

Part of the uptake of benzanthrone by the skin could also be attributed to lipids present in the skin, as is evident from the binding of benzanthroneltoskin lipids micelles *in vitro*.

On the basis of the above preliminary investigation, it may be assumed that benzanthrone due to its lipophilic nature is readily absorbed through the skin preferably crossing the lipoidal barrier of the epidermis and gets bound to certain specific sites in the skin. The skin-bound benzanthrone is subsequently released into circulation.

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Study of sodium homeostasis in blood in whole body gamma-irradiated rats

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Abstract. Adult male rats were subjected to whole body irradiation of 400 and 1000 rads and sodium homeostasis in blood was studied on the 1st, 4th and 7th days after exposure. A significant decrease in red cell adenosine triphosphatase, progressive loss of erythrocytic potassium and alteration in influx and efflux of sodium-22 in red cells *in vitro* were observed in these rats.

Keywords. Whole body irradiation; influx and efflux of sodium-22; erythrocytic ATPase.

Introduction

The cellular lethality induced by ionising radiation is probably mediated by membrane damge (Myers, 1970). It has been postulated that alteration of membrane permeability plays an important role in radiation damage at the cellular level (Bacq *et al.*, 1961; Sutherland *et al.*, 1967). Red blood cells are an excellent model for membrane transport studies. Knowledge of the characteristics of sodium and potassium fluxes across the cell membrane has accumulated to a great extent from studies on red cells (Caviers, 1977). Many studies (Sutherland *et al.*, 1967; Bresceani *et al.*, 1964; Cook, 1965; Nakken, 1966; Killman *et al.*, 1969; Misra *et al.*, 1980) have been carried out on the effects of X-irradiation on the transport of sodium and potassium in erythrocyte membranes. Most of the previous studies were carried out *in vitro* on isolated, irradiated red cell suspensions and the mechanism of radiation-induced changes in cells had not been fully elucidated. The present paper reports the effects of sublethal and lethal doses of whole body γ-irradiation on sodium homeostasis as revealed by influx and efflux of sodium-22 as sodium chloride in red cells *in vitro* in experimental rats.

Materials and methods

Adult male Sprague Dawley rats were subjected to sublethal and lethal doses of whole body γ-irradiation of 400 and 1000 rads respectively (dose rate—100 rads/min), from a ^{60} cobalt source, Model GC 200, Atomic Energy of Canada Ltd., Ottawa, Canada. Experiments were done on 1st, 4th and 7th days after exposure, for a better understanding of the repair and recovery mechanism in the damaged tissues in irradiated animals. Experiments could not be carried out beyond the 7th day in rats exposed to 400 rads due to non-availability of enough animals. Influx and efflux of sodium in erythrocytes *in vitro* using sodium-22 was studied as reported (Dunn *et al.*, 1969a) using Nuclear Chicago Autogamma Spectrometer Model 4219. Adenosine triphosphatase (ATPase EC 3.6.1.4) activity was studied by the recommended method (Dunn *et al.*, 1969b). Sodium (Weinbach, 1935) and potassium (Gupta, 1967) in plasma and red cells were estimated colorimetrically.

Results and discussion

The influx and efflux of sodium-22 in red cells (*in vitro*) of both groups of experimental rats remained unaltered (figures 1 and 2) 24 h after irradiation. Erythrocytes of rats after the 4th day of exposure to 400 rads showed a decrease ($p<0.01$) in influx of sodium-22 followed by an increased ($p<0.01$) influx on the 7th day. In contrast, rats exposed to 1000 rads showed increased ($p<0.01$) incorporation of sodium-22 in red cells after the 4th day of exposure.

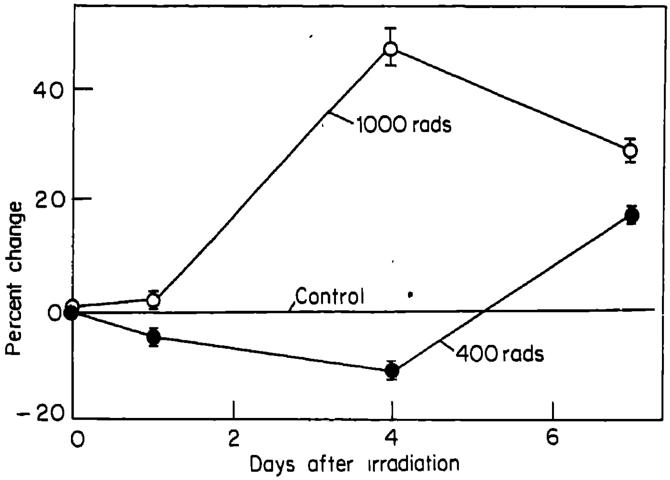

Figure 1. Whole body γ-irradiation and influx of sodium-22 in red cell (*in vitro*). (Result at each point is based on 6 to 8 rats)

Efflux of sodium-22 in red cells of experimental groups of rats was initially decreased ($p<0.001$) on the 4th day followed by a significant increase over the control on the 7th day. After the receipt of radiation (figure 2) the red cell sodium content in rats exposed to 400 rads was the same as control throughout (table 1). Rats with 1000 rads exposure showed loss in red cell sodium from 4th day onwards. Concentration of plasma sodium in rats exposed to 400 rads declined from the 1st to the 7th day after exposure. Rats exposed to 1000 rads did not show any change in plasma sodium except a decrease on the 7th day after exposure. A progressive decrease in red cell potassium has been observed in post irradiation periods in both the groups of experimental rats, while plasma potassium of treated rats was

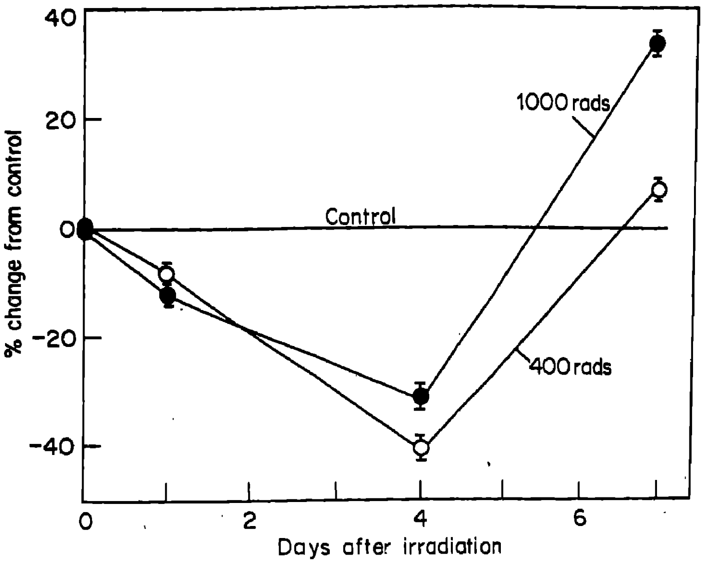

Figure 2. Whole body γ-irradiation and efflux of sodium-22 in red cell (*in vitro*) (Result at each point is based on 6 to 8 rats)

Table 1. Effect of whole body γ-irradiation on the levels of blood sodium and potassium in rats.

Group	Plasma sodium (meq/litre plasma)	Red cell sodium (meq/litre plasma)	Plasma potassium (meq/litre plasma)	Red cell potassium (meq/litre cells)
Control	133.2±1.6	24.8±0.8	7.3±0.1	39.9±0.7
Experimental (I) (1st day after exposure)				
400 rads	120.5±4.4[a]	28.4±1.2	7.0±0.4	35.1±1.2[a]
1000 rads	141.4±5.8	24.5±1.6	7.0±0.2	36.0±1.4[b]
Experimental (II) (4th day after exposure)				
400 rads	119.7±1.4[a]	25.5±1.7	7.5±1.2	29.6±2.8[c]
1000 rads	131.3±2.7	20.4±1.6[a]	7.6±1.3	32.4±2.1[c]
Experimental (III) (7th day after exposure)				
400 rads	126.6±2.0[d]	21.9±0.8	7.6±0.3	26.5±2.1[c]
1000 rads	120.5±3.0[a]	20.4±1.9[a]	7.4±1.9	30.8±2.1[c]

(Values are Mean ±SE of 6-8 rats)

[a] $p<0.01$; [b] $p<0.02$; [c] $p<0.001$; [d] $p<0.05$.

same as the control, throughout (table 1). Twentyfour hours after exposure, red cell ATPase (basal) activity of both groups of experimental rats was decreased by 50%, (figure 3). In rats exposed to 400 rads,ATPase (basal) levels became normal on the 4th day of exposure, while the activity of the enzyme in rats exposed to irradiation of 1000 rads reached the control value on the 7th day only. Sodium, potassium activated ATPase in red cells of rats was decreased more than 60% on the 1st day after exposure to radiation. Thereafter, the enzyme increased progressively in both the groups of rats and it never reached control values even after exposure for 7 days (figure 3). Both groups of experimental rats, 24 h after exposure, showed more than 65% inhibition of ouabain-sensitive red cell ATPase activity, followed by progressive increase in the enzyme activity, though it could not reach the control value till the 7th day of exposure (figure 3).

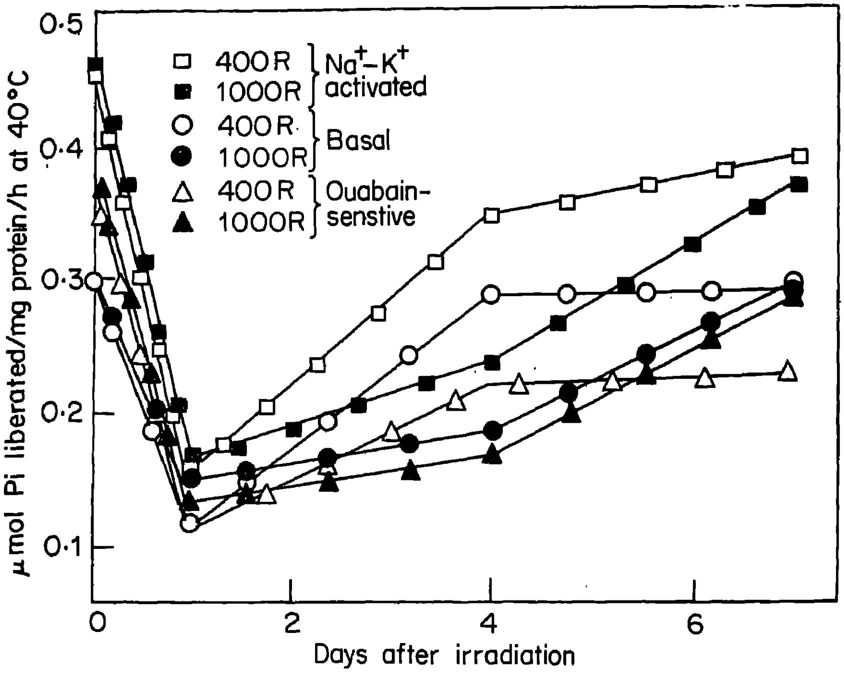

Figure 3. Whole body γ-irradiation and red cell ATPase.

Results of the present investigation show that sub-lethal and lethal doses of whole body irradiation do not affect red cell sodium homeostasis at the initial stage of treatment, though ATPase activities were significantly decreased on the 1st day of exposure (figure 3). Significant loss of red cell potassium in rats observed at all stages after irradiation are in agreement with the previous findings (Sutherland *et al.*, 1967; Bresceani *et al.*, 1964; Cook, 1965; Nakken, 1966; Killman *et al.*, 1969; Misra *et al.*, 1980) based on *in vitro* exposure of red cells to radiation.

Results of influx and efflux of sodium-22 in erythrocytes of irradiated rats (figures 1 and 2) suggest an alteration in sodium transport, affected by ionizing radiation, part of which could be due to an altered pumping mechanism in the membrane (Bresceani *et al.,* 1964). Changes in the concentration of Na^+ and K^+ions, observed in a certain stage of whole body exposure could be attributed partly to passive ion movement, resulting from alteration in the membrane structure (Myers *et al.,* 1966). Significant inhibition of red cell ATPase (basal) activity induced by whole body irradiation is not in agreement with the findings of Bresciani *et al.* (1964), who observed minor changes in enzyme in isolated irradiated human red cell, while the observed changes in sodium and potassium activated ATPase are comparable with their findings.

The present results need not conform to the *in vitro* findings of Bresciani *et al.* (1964), as *in vitro* irradiation of erythrocytes causes damage to cellular membrane *per se.* The present findings may be attributed to secondary effects of whole body irradiation. This is the main reason why effective radiation doses for *in vitro* and *in vivo* studies are different. The observed response of ouabain-sensitive ATPase in whole body irradiated rats suggest recovery of radiation damage at enzyme level even after 7 days of post irradiation period. More detailed studies are required for elucidation of radiation injury at the cellular level and the recovery process.

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Metabolism of echitamine and plumbagin in rats

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Abstract. When administered to rats, echitamine was absorbed rapidly from the tissues and was detected in circulation within 30 min. The drug level reached a maximum value by 2 h and then decreased steadily. The drug had completely disappeared from the blood in 6 h. The presence of echitamine was observed within 2 h in urine and the maximum amount of drug was excreted between 2 and 4 h. About 90% of the drug was excreted in urine in 24 h and the drug could not be detected in urine after 48 h. Along with echitamine, its metabolites were also detected in the urine. Plumbagin was not detected in blood upto 24 h when administered into rats. The drug was detected in urine within 4 h after administration; a major portion of the drug was excreted in urine by 24 h and traces of the drug were observed in the urine even after 48 h.

Keywords. Echitamine; plumbagin; pharmacokinetics; urinary metabolites; plant extracts.

Introduction

During the past 25 years, chemotherapy has gained an important role in the treatment of tumours. Plants are a suitable source for they provide chemicals with novel structures. So far, many plant products have been found to be active antitumour agents both in animal tumour systems and human tumours (Hartwell, 1976). Preliminary studies with echitamine, an indole alkaloid, extracted from, *Alstonia scholaris* and plumbagin, a naphthoquinone derivative (Chandrasekaran and Nagarajan, unpublished observations; Krishnaswamy and Purushothaman, 1980) from *Plumbago zeylanica* were reported to have antitumour activity on rat fibrosarcoma. Simple and sensitive methods have been developed for the detection and quantitative estimation of echitamine and plumbagin in biological samples (Chandrasekaran *et al.*, 1981). Information on the metabolism and subsequent excretion of these drugs is of particular importance, since serious and unexpected toxicity can arise from accumulation of a drug normally detoxified by the liver and/or excreted by the kidney in patients with compromised hepatic or renal function (Sieber *et al.*, 1976). In this paper, we report the metabolic fate and excretory pattern of echitamine and plumbagin in rats.

Materials and methods

Echitamine and plumbagin were generous gifts from Dr K. K. Purushothaman of Captain Srinivasamurthi Research Institute, Madras. Echitamine used was in the form of echitamine chloride. Echitamine (2.8 mg/0.2 ml) or plumbagin (1.6 mg 0.2 ml) was injected intramuscularly in the thigh region to rats.

A total number of 48 male Wistar rats (about 100 g) were injected with the drug and divided into 16 groups of 3 each. Blood samples were drawn into heparinized tubes from 3 rats at different time intervals from 0-24 h by heart puncture. Plasma was separated and used for estimation of drugs under study. Plasma samples containing high content of lipid were excluded for the estimation of drug since the removal of lipid from plasma sample resulted in loss of drug also. Urine samples were collected in metabolic cages from drug injected rats (3) at the intervals of 2, 4, 6, 24 and 48 h. Two ml of toluene was used as preservative for urine. Control urines were collected from the normal rats in a similar manner.

Estimation of drugs in blood

Echitamine: Plasma (0.5 ml) was deproteinized by adding 1 ml of 10% sodium tungstate followed by 1 ml of 0.66 N suphuric acid, the resultant protein-free supernatant adjusted to 20% NaOH concentration and extracted with 10 volumes of dioxan. The dioxan extract was dried *in vacuo* and dissolved 1 ml of water. The blue colour developed on the addition of 0.66 N phenol reagent (1 ml) (Folin and Ciocalteau, 1927) followed by 20% Na_2CO_3 (1 ml) was read at 700 nm in a Carlzeiss JENA VSU 2 spectrophotometer, 15 min after addition of the reagents. The recovery was about 70%. Blood collected just prior to the administration of the drug (0 h) was treated as control.

Plumbagin: Plasma (0.5 ml) was treated with 1 ml of 10% sodium tungstate, 1 ml of 0.66 N sulphuric acid and 1 ml of 10% NaOH. The mixture was heated at 80°C for 30 min, cooled to room temperature (30°C) and centrifuged (1000 *g*) in a Remi T_8 centrifuge. The clear supernatant was separated and the absorbance was measured at 500 nm. The recovery of the added drug was about 80%.

Metabolic studies

Echitamine: Pooled urine sample from 3 treated rats (100 ml) was concentrated *in vacuo* to about 3 ml, mixed with an equal quantity of 40% NaOH and extracted with six vol of dioxan. The dioxan layer was separated and dried *in vacuo* and the dried mass dissolved in 1 ml of water was chromatographed using a Whatman 3 MM paper in duplicate along with reference echitamine and 'control-urine' extract using butanol: citric acid: water (87:0.48:13, v/w/v) and butanol: acetic acid: water (67:10:23, v/v) as solvent systems. The chromatograms were sprayed with 0.66 N phenol reagent followed by 20% Na_2CO_3 or Dragendorff reagent (Clarke, 1974). Blue and orange colour were developed with phenol and Dragedorff reagents respectively on reaction with echitamine. Blank regions of the chromatograms similarly processed served as control.

Plumbagin: The pooled urine sample (100 ml) from drug treated rats was concentrated *in vacuo.* The concentrated material was acidified to pH 4.0 and extracted with 6 vol of chloroform and concentrated to 0.5 ml. Chloroform extract (10 µl) was chromatographed on thin layer silica gels using hexanebenzene solvent system (1:1, v/v). Plumbagin was detected by exposing the plates either to iodine or bromine vapours.

To the vacuum dried aliquots of chloroform extract (20-80 µl),1 ml of 10% NaOH was added followed by 2 ml water. The tubes were kept at 80°C for 30 min, cooled and the intensity of the colour developed was measured at 500 nm. Urine control and standards were studied in a similar manner.

Results and discussion

The presence of echitamine could be detected in blood within 30 min of its administration. The drug level steadily increased upto 2 h and then decreased gradually and at the end of 6 h, the drug could not be detected in blood (figure 1).

Analysis of urine at different time intervals (2, 4, 6, 24 and 48 h) after administration of echitamine showed the presence of echitamine in all the samples. Chromatography of the dioxan extract from urine in butanol, citric acid solvent system showed the presence of echitamine alone (Rf, 0.8) at 2 and 4 h, but in subsequent samples, the presence of two compounds with Rf 0.25 and 0.1 were observed along with echitamine. Separation by butanol, acetic acid solvent

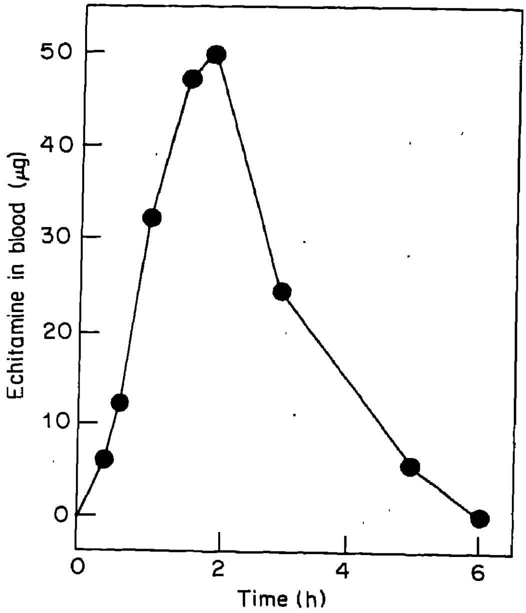

Figure 1. Time course of appearance of echitamine in blood.

Each rat was injected 2.8 mg of the drug intramuscularly and blood was collected at time intervals indicated in the figure by cardiac puncture and echitamine present was estimated.

system showed only one compound (Rf, 0.7) which corresponded to automa echitamine (table 1). The compounds with Rf value 0.25 and 0.1 were consider as metabolites of echitamine since none of the metabolites from control uri developed colour with Dragendorff reagent under the experimental conditio

Table 1. Paper chromatographic separation of echitamine and its metabolites.

Compound	Echitamine treated		Plumbagin treated
	Solvent systems a	b	Solvent system c
	Rf		Rf
Echitamine	0.8	0.7	
Compound A*	0.25	N.D	
Compound B*	0.1	N.D	
Plumbagin			0.6

(a) Butanol: citric acid: water (87:0.48:13 v/w/v);

(b) Butanol: acetic acid: water (67:10:23, v/v) solvent system. Thin layer chromatographic separation of plumbagin in (c) hexane: benzene (1:1 v/v) solvent system from urine extracts of rats after drug administration.

* A and B are metabolites of echitamine and the chemical nature of these compounds are yet tc be characterized.

N.D. Metabolites of echitamine A and B were not detectable in this solvent system.

However, the chemical structures of these compounds are yet to be characterize Excretion of the drug was maximum between 2 and 4 h. Eighty per cent of the dr administered was excreted unchanged. About 90% of the drug and its metaboli were excreted on the 1st day and the remaining portion was excreted on the 2 day. The drug was not detectable in the urine after 48 h. Excretion of echitami at different time intervals has been shown in table 2. The excretory patterns echitamine in normal rats and tumour-bearing rats were similar.

Table 2. Excretion of echitamine and plumbagin in urine of rats.

Compound	Amount excreted (μg) Hours 0-2	2-4	4-6	6-24	24-48
Echitamine	220	860	430	520	240
Plumbagin	—	60	110	320	180

Values represent mean values from 6 rats, 3 in each set. Amount of echitamine injected—2.8 mg to each rat. Amount of plumbagin injected—1.6 mg to each rat.

Even though phenol reagent is sensitive, it is not specific for the drug and its metabolites. Some of the normal metabolites from control urine were extracted into dioxan and developed blue colour with phenol reagent, whereas Dragendorff reagent was specific for the drug under the experimental conditions. However, the latter was not useful when the drug was present in lower concentrations.

Administration of plumbagin into rats and subsequent collection of blood at different time intervals did not show its presence in any sample. It could be postulated that the drug might have been extensively distributed in body tissues with a very small level in the blood and thus its detection in the blood would not be possible with the current methods. Alternatively, there may also be an unidentified compound present in the blood (cellular and/or humoral component, nonfilterable by the kidneys) which could possibly interfere with the detection of plumbagin in the blood.

Analysis of urine at different time intervals after administration of plumbagin showed the presence of plumbagin in all the samples except 2 h urine (table 2). Separation of a chloroform extract by thing layer chromatography showed the presence of one spot (Rf, 0.6) in benzene: hexane (1:1, v/v) solvent system which corresponded to authentic plumbagin (table 1).

A considerable amount of plumbagin was lost during its extraction from urine. So the extraction method was modified by taking aliquots of urine collected from plumbagin-injected rats and 1 ml of 10% NaOH was added and the final volume made upto 3 ml with water; the colour developed had an absorption peak at 490 nm. This method is useful only if the concentration of plumbagin is above 5 µg in urine and it is free from turbidity. It was observed that none of the normal metabolites extracted into chloroform developed colour similar to plumbagin with 10% NaOH.

The contents of duodenum and small intestines of rats were washed with normal saline and analyzed for plumbagin after administration of the drug using the extraction procedure described for urine. The presence of the drug was observed after 6 h. Attempts made to quantitate the drug were not successful due to the interference with other compounds present, but it was apparent that considerable amount of plumbagin was excreated in faeces.

Echitamine is absorbed rapidly from the tissues into blood and cleared from the systemic blood in a short period. The injected drug is almost completely excreted in urine within 48 h. Similarly a major portion of plumbagin was excreted in urine and faeces within 48 h, questioning the direct lethal effect of echitamine on tumours. On the other hand, the antitumour effect reported may be due to the triggering of some reaction by the parent compound. Experiments are under way to isolate the metabolic products when echitamine is incubated with liver slices *in vitro*.

Acknowledgements

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In vitro effect of prostaglandins, prostaglandin endoperoxides and thromboxane on rat intestinal alkaline phosphatase and ($Ca^{2+}-Mg^{2+}$) adenosine triphosphatase

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Abstract. The activity of alkaline phosphate and $Ca^{2+}-Mg^{2+}$ adenosine triphosphatase, two of the enzymes involved in lipid and calcium uptake across the intestinal membrane, were increased in experimental atherosclerosis. Administration of *Annapavala sindhooram*, an antiatherosclerotic drug, lowers these enzyme levels to near normal values. Prostaglandin E_2 stimulated the enzyme activities *in vitro*, while prostaglandin endoperoxide inhibited the activity. Thromboxane and other prostaglandins had no effect on the enzyme activities. Addition of the antiatherosclerotic drug to the *in vitro* assay system reversed the effect of both prostaglandin E_2 and prostaglandin endoperoxide.

Keywords. Prostaglandins; prostaglandin endoperoxide; thromboxane; alkaline phosphatase; $Ca^{2+}-Mg^{2+}$ ATPase; atherosclerosis; hypolipaemic drug.

Introduction

Song and Adham (1978, 1979) showed that the transport of zinc ions in the intestine was facilitated by prostaglandins. Several transport mechanisms in the intestine involve alkaline phosphatase (EC 3.1.3.1.), a zinc containing enzyme (Flock and Bollman, 1948; Madsen and Tuba, 1952; Jensen, 1979; Lie *et al.*, 1979); and both alkaline phosphatase and $Ca^{2+}-Mg^{2+}$ ATPase (EC 3.6.1.3) are reported to be the functional expression of a single protein (Haussler *et al.*, 1970; Norman *et al.*, 1970; Russel *et al.*, 1972). Prokaryotes, like *Micrococcus sodonensis*, contain only Ca^{2+} ions as a component of alkaline phosphatase (Colew and Heath, 1971) and recently, a calcium ion stimulated alkaline phosphatase was identified in synaptic vesicles,(Zisapel and Levi, 1980).Alkaline phosphatase and $Ca^{2+}-Mg^{2+}$ ATPase activities are known to be affected in a variety of pathological conditions, such as diabetes, hyperlipaemia and the resultant atherosclerotic disease (Pillai, 1978; Simon, 1978; Sunitha and Shanmugasundaram, 1980). In our laboratory, increased activity of intestinal alkaline phosphatase and other ATPase were observed in diabetic and hypercholestrolemic rabbits (Sunitha and Shanmugasundaram, 1980).

Abbreviations used: APS, *Annaparala sindhooram*; PG, Prostaglandins; TxB, Thromboxane.

The effect of a plasma lipid lowering agent, *Annapavala sindhooram* (APS) prepared in our laboratory (based on the principles of Siddha medicinal system) was previously shown to have hypolipaemic effects in rats, rabbits and man (Marita *et al.*, 1978; Shanmugasundaram and Parthasarathy, 1981). APS is a herbomineral preparation containing green vitreol and coral reef as the basic minerals and *Aealypha indica, Lippia nodiflora, Vinca rosea, Lawsonia alba, Hibiscus rosasinesin, Phyllanthus emblica* and *Cyanadon dactylon* as the herbal ingredients. (The mineral composition is as follows: Fe_2O_3, 22-25%; CaO, 35-38%; MgO, 5-6%; MnO, 0.1-0.2%; no detectable zinc, mercury or lead; sulphates, 18-19%; phosphate (P_2O_5), 1-1.5%; SiO_2, 3.5-4%, CuO, 50-60 parts per million; traces of iodine.) In this communication, we report the effect of prostaglandins (PG) on the intestinal alkaline phosphatase and $Ca^{2+}-Mg^{2+}$ ATPase in normal and atherosclerotic rats. The intestine of rats which were made atherosclerotic by dietary modification, and then fed on *A. sindhooram* extract.

Materials and methods

Male Wistar rats weighing 80-100 g were fed on commercial pelleted rat chow marketed by M/s. Hindustan Lever, Bombay, and water was provided *ad libitum*. Hyperlipaemia was induced in rats by feeding an atherogenic diet based on the formula of Hahn *et al.* (1977) with minor modifications.

Animals were divided into three groups and were maintained as follows: Group 1: animals on normal diet for four weeks had a serum cholesterol level of 77±5.8 mg/100 ml; Group 2: animals maintained on an atherogenic diet for four weeks which had a serum cholesterol level of 360 ±9.9 mg/100 ml at the end of the experimental period and in which the aorta showed focal areas of degeneration and gross lipid inclusions in both the intima and media; Group 3: animals maintained on atherogenic diet for four weeks followed by normal diet and *A. sindhooram* extract (30.0 mg/day) for four weeks when the serum cholesterol level was 91 ±6.2 mg/100 ml in this period, the rat aorta showed considerable decrease in the lipid deposits (more than 75% regression) and the cells showed no focal degeneration.

At the end of the experimental period, animals were killed by cervical decapitation. Intestine (proximal 10-12 cm of small intestine) was quickly removed, washed with ice-cold saline and rinsed with ice-cold 5 mM EGTA (pH 7.4) slit lengthwise and the mucosa was scrapped with a glass slide. The brush border membrane preparations were made from the scrapings, according to the method of Forstner *et al.* (1968) as modified by Melancon and DeLuca (1970). Alkaline phosphatase activity was determined in the brush border by the method described by King (1965) as modified by Ramadoss *et al.* (1977) using disodium phenyl phosphate as substrate and $Ca^{2+}-Mg^{2+}$ ATPase was estimated by the method described by Reddy (1972). Protein was estimated by the method of Lowry *et al.* (1951) as modified by Markewell *et al.* (1978).

Prostaglandins A_1, A_2, B_1, B_2, E_1, E_2, $F_1\alpha$ and $F_2\alpha$, thromboxane B_2 (TxB_2) and prostaglanding endoperoxides, U_{46619} and U_{44069} were kind gifts from Dr. J. E. Pike of

UpJohn Company, Kalamazoo, Michigan, USA. Prostaglandins, thromboxane and the endoperoxides (50 µg each) dissolved in 0.05 ml of ethanol was added to the reaction systems (3.0 ml), and alkaline phosphatase and $Ca^{2+}-Mg^{2+}$ ATPase activities were assayed. The assays were repeated with the inclusion of 1 mg. *A. sindhooram* in addition to prostaglandins in the reaction system.

Results and discussion

Table 1 depicts the changes due to the *in vitro* addition of prostaglandins on the activity of alkaline phosphatase in the intestine of normal, hyperlipaemic and *A. sindhooram*-treated rats. It was observed that intestinal alkaline phosphatase activity was elevated in the hyperlipaemic state and that this was reduced by *A. sindhooram* administration. The presence of PGE_2 in the reaction system increased the alkaline phosphatase activity in all the three experimental conditions studied. It is significant that addition of *A. sindhooram* to the reaction mixture prevented the stimulatory effect of PGE_2, although in the *A. sindhooram*-treated animals, the intestinal enzyme was stimulated by the addition of PGE_2 and behaved like the normal enzyme. From table 2, it could be seen that prostaglandin endoperoxide, U_{44069}, inhibited the alkaline phosphatase activity in the normal rat intestine and *A. sindhooram* addition reversed this trend. However, U_{44069} had no effect on the alkaline phosphatase in the hyperlipaemic and *A. sindhooram*-treated animals.

Table 1. Effect of prostaglandins on the alkaline phosphatase activity *in vitro* in normal hyperlipaemic and APS treated rat intestine.

	Normal intestine		Hyperlipaemic intestine		APS-treated intestine (*in vivo*)
	Without APS	With APS	Without APS	With APS	
Control	952±61	933±58	1379±82	1352±81	1101±76
PGA_1	994±62	962±62	1337±80	1390±86	1062±69
PGA_2	969±63	976±58	1349±72	1379±80	1156±60
PGB_1	976±63	981±61	1363±82	1398±82	1098±72
PGB_2	992±62	990±63	1356±83	1384±79	1171±62
PGE_1	960±61	962±61	1372±81	1396±82	1128±76
PGE_2	1109±59[a]	1000±59	1499±88[b]	1382±83	1193±57[b]
$PGF_1\alpha$	999±63	969±61	1340±81	1390±78	1096±66
$PGF_2\alpha$	1010±59	1019±63	1360±84	1382±81	1163±74

The enzyme activity was assayed in the normal and hyperlipaemic conditions with and without added APS. The values were the mean of five animals in each group. The activity is expressed as nano mols of phenol liberated in 15 min per mg protein.

[a] $p<0.01$, [b] $p<0.1$.

Table 2. Effect of thromboxane and prostaglandin endoperoxides on the alkaline phosphatase activity *in vitro* in normal, hyperlipaemic and APS-treated rat intestine.

Compounds	Normal intestine		Hyperlipaemic intestine		APS-treated intestine (*in vivo*)
	Without APS	With APS	Without APS	With APS	
Thromboxane TxB$_2$	980±61	970±62	1373±79	1399±80	1152±75
Prostaglandin, endoperoxide					
U$_{46619}$	983±61	998±62	1366±80	1394±82	1148±70
U$_{44069}$	811±60[a]	969±64	1359±81	1390±83	1139±71

The results were the mean of five animals in each group. The activity is expressed as nano mol of phenol liberated in 15 min per mg protein (Mean ±S.D.).

[a] $p<0.02$.

$Ca^{2+}-Mg^{2+}$ ATPase activity was not affected by any of the prostaglandins (table 3) but in the presence of *A. sindhooram,* PGE$_2$ increased the activity ($p<0.001$). However in the atherosclerotic rat intestine, PGE$_2$ stimulated $Ca^{2+}-Mg^{2+}$ ATPase activity ($p<0.01$) and this was restored to normal level by the addition of *A. sindhooram* to the reaction mixture. In the treated animals, PGE$_2$ also stimulated $Ca^{2+}-Mg^{2+}$ ATPase activity. Prostaglandin endoperoxide, U$_{44069}$, inhibited $Ca^{2+}-Mg^{2+}$ ATPase activity in normal and hyperlipaemic

Table 3. Effect of prostaglandins on the $Ca^{2+}-Mg^{2+}$ ATPase activity *in vitro* in normal, hyperlipaemic and APS treated rat intestine.

Compounds	Normal intestine		Hyperlipaemic intestine		APS treated intestine (*in vivo*)
	Without APS	With APS	Without APS	With APS	
Control	2.6±0.4	2.2±0.3	3.4±0.4	3.1±0.3	2.9±0.4
PGA$_1$	2.3±0.3	2.5±0.4	3.1±0.4	2.7±0.3	2.7±0.3
PGA$_2$	2.2±0.4	2.6±0.3	3.6±0.3	2.8±0.4	3.1±0.4
PGB$_1$	2.7±0.3	2.7±0.3	3.5±0.4	3.1±0.3	2.8±0.3
PGB$_2$	2.2±0.4	2.8±0.4	3.2±0.4	2.9±0.3	3.3±0.3
PGE$_1$	2.6±0.3	2.2±0.3	3.4±0.3	3.0±0.4	3.6±0.3[b]
PGE$_2$	3.0±0.4	3.9±0.4[a]	4.7±0.5[b]	3.3±0.3	3.8±0.4[c]
PGF$_{1\alpha}$	2.9±0.4	2.6±0.3	3.3±0.4	2.8±0.4	2.7±0.4
PGF$_{2\alpha}$	2.6±0.3	2.9±0.4	3.5±0.3	2.7±0.3	2.8±0.3

The results were the mean of five animals in each group. The activity is expressed as μmol of phosphorus liberated in 15 min per mg protein (Mean ±S.D.).

[a] $p<0.001$; [b] $p<0.01$. [c] $p<0.02$.

intestine (table 4). In the presence of *A. sindhooram,* the prostaglandins were without effect on the two enzymes tested, indicating that this material prevented the alterations caused by PGE_2 and PG endoperoxide U_{44069} in the animals fed with atherogenic diet.

Table 4. Effect of thromboxane and prostaglandin endoperoxides on the $Ca^{2+}-Mg^{2+}$ ATPase activity *in vitro* in normal, hyperlipaemic and APS treated rat intestine.

Compounds	Normal intestine		Hyperlipaemic intestine		APS treated intestine (*in vivo*)
	Without APS	With APS	Without APS	With APS	
Thromboxane	2.4±0.3	2.7±0.3	3.4±0.3	3.2±0.4	3.2±0.4
Prostaglandin endoperoxide					
U_{46619}	2.6±0.3	2.8±0.4	3.2±0.4	3.1±0.4	2.9±0.3
U_{44069}	1.1±0.2[a]	2.6±0.3	2.4±0.4[b]	2.9±0.3	3.3±0.3

The results were the mean of five animals in each group. The activity is expressed as μ mol of phosphorus liberated in 15 min per mg protein (Mean ±S.D.).

[a] $p<0.001$; [b] $p<0.01$.

Several investigators have observed stimulation of alkaline phosphatase and $Ca^{2+}-Mg^{2+}$-activated ATPase by PGE_2 (Mirham *et al.,* 1977) Sandhya and Das, 1980; Gilbert and Wyllie, 1979; Otsuka *et al.,* 1980; Acker *et al.,* (1980). The effects reported here could be due to complexing of PGE_2 by the metal ion present in the plant material. In addition, the decreased levels of lipids noticed on administration of *A. sindhooram* could also be due to the cumulative effect of different metal ions present in the material on the uptake of lipids and bile acids in the enterohepatic recirculation.

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Effect of denaturants on the oligomeric structure of the 11 S protein of sunflower *(Helianthus annuus)*

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Abstract. The effect of sodium dodecyl sulphate, urea, guanidinium hydrochloride and heat on the oligomeric structure of the 11 S protein of sunflower has been determined. Sodium dodecyl sulphate directly dissociates the protein to 2 S subunits, whereas urea and guanidinium hydrochloride dissociate it through an intermediate 7 S protein. Heating the protein at 90°C for 20 min caused dissociation of the 11 S protein, without any precipitation.

Keywords. *Helianthus annuus* (sunflower); 11 S protein; urea; guanidinium hydrochloride; heat denaturation; oligomeric structure.

Introduction

The proteins of sunflower consist of four fractions designated by sedimentation coefficients of 1.7, 7.8, 11.9 and 18.1 S (Schwenke *et al.*, 1974); the 11.9 S fraction is the major protein. This protein has been fractionated to homogeneity (Joubert, 1955; Schwenke *et al.*, 1974; Rahma and Narasinga Rao, 1981). Its molecular weight is 300,000. On SDS-PAGE, it shows at least 10 polypeptide chains showing that it is a high mol. wt. protein consisting of subunits. Further, it consists essentially of β-structure and random coil and has very little α-helical structure (Rahma and Narasinga Rao, 1981). Many oilseed proteins possess these characteristic features of high mol. wt. and very little α-helical structure. Detailed studies have been made on the effect of denaturants on proteins which have a fair or high proportion of α-helical structure (Tanford, 1968). Such studies on isolated oilseed proteins have been limited and scanty. In this laboratory, we have started a systematic study on the effect of denaturants on oilseed proteins. As a part of this investigation, we have studied the effect of sodium dodecyl sulphate (SDS), urea, guanidinium hydrochloride (GuHCl) and heat on the oligomeric structure of the 11 S protein of sunflower seeds.

Abbreviations used: mol. wt., Molecular weight; SDS.sodium dodecyl sulphate; GuHCl, guanidinium hydrochloride; PAGE, polyacrylamide gel electrophoresis.

Although the exact $S_{20^\circ w}$ value of the protein is 11.9 S, it is usually referred to
11 S protein of sunflower seed and we use the same nomenclature.

Materials and methods

Sunflower

Russian variety EC 68414, grown in the state of Karnataka, was obtained from loc
market.

Isolation of 11 S protein

11S protein was prepared by the method described earlier (Rahma and Narasin
Rao, 1981). It was homogeneous as judged by gel filtration, gel electrophore
and sedimentation velocity techniques.

Sedimentation velocity

The experiment was done using 1% protein solution in the appropriate solvent
room temperature (~28°C) at 59,780 rpm in a Spinco Model E analytical ultr
centrifuge equipped with a rotor temperature indicator and control unit and phas
plate schlieren optics. From the photographs taken at different intervals
centrifugation, the sedimentation constant was calculated and corrected for t
viscosity of the solvent.

The measurements were made in 0.025 M tris-glycine buffer, pH 8.3, containi
various concentrations of SDS, urea or GuHCl.

Heat denaturation

Five ml of 0.5% protein solution in 0.025 M tris-glycine buffer of pH 8.3 were heat
for 10 min at various temperatures, in the range 30-90°C, in a thermostatic wa
bath. For each temp, a separate aliquot was used. The protein solution was th
cooled immediately in an ice bath and rewarmed to room temp. The turbidity
the solution was measured by determining the transmittance at 540 nm in
Spectronic 20 spectrophotometer. From the transmittance value, a quant
$(T_0 - T/T_0)\times 100$, was calculated where, T_0 is the transmittance of the unheat
sample and T, that of the heated sample. The heated samples were examined
polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide gel electrophoresis

This was carried out by the procedure of Davis (1964) using 7.5% gels. T
experiments were done in 0.025 M tris-glycine buffer, pH 8.3. The gels w
stained with 0.5% amido black in 7.5% acetic acid for 1 h and then destined.

The pH measurements were made with a Radiometer pH meter TTT2 a
transmittance measurements with Spectronic 20.

Results and discussion

The dissociative effects of SDS, urea and GuHCl on the 11 S protein of sunflov
were followed by the sedimentation velocity technique. It was observed that
effect was time dependent upto 3 h; after this interval of time, no further chan

were observed. Therefore, in all the cases, the solution were left at room temp (~28°C) for 3 h after the addition of the denaturant and measurements made subsequently.

The 11 S protein gave a single symmetrical peak with $S_{20,w}$ value of 11.6 S at 1% protein concentration (figure 1A). The same result was obtained whether the measurements were made in 1 M NaCl or 0.025 M tris-glycine buffer, pH 8.3; apparently, salt had no associative or dissociative effect on the protein.

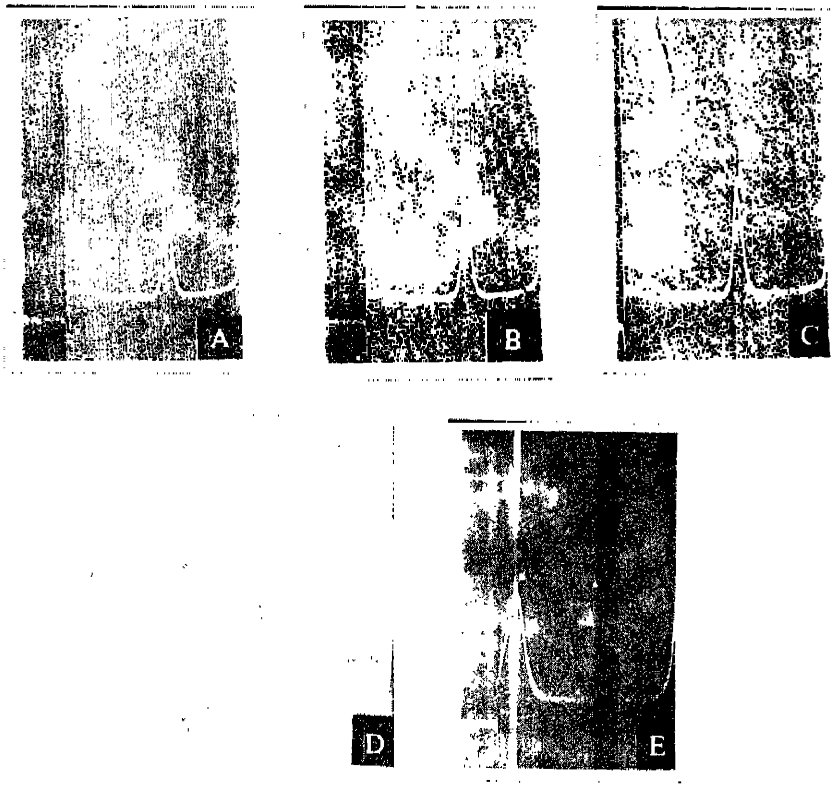

Figure 1. Sedimentation velocity pattern of 11 S protein in the presence of SDS (0.025 M tris-glycine buffer, pH 8.3).

A. 11 S protein; B. 11 S protein +0.05% SDS; C. 11 S protein +0.10% SDS; D. 11 S protein +0.30% SDS; E. 11 S protein +1.50% SDS. (Sedimentation proceeds from left to right).

The addition of SDS upto 0.05% caused no observable change in the sedimentation on velocity pattern (figure 1B). However, at higher concentrations, two peaks with S_{app} values of 11 S and 2 S were observed; the proportion of the latter increased with increase in SDS concentration (figure 1C, D). At 1.5% SDS concentration, the 2 S peak alone was observed (figure 1E). Apart from the 11 S and 2 S proteins no other protein was observed at any SDS concentration.

From the enlarged tracings of the sedimentation velocity patterns, the proportions of the two peaks at various SDS concentrations were calculated and are given in figure 2. Where the peak was not well-separated from the meniscus, a symmetrical peak was drawn and the area measured. The areas were not corrected for radial dilution. Because of these limitations, quantitative significance could not be attached to the proportion of the proteins in the mixture. At and above 1.5% SDS concentration, dissociation of the oligomeric protein to 2 S protein appeared to be complete.

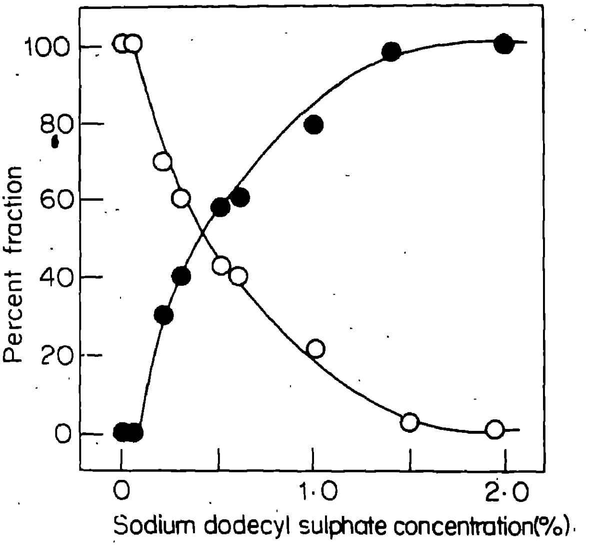

Figure 2. The proportion of protein fraction as a function of SDS concentration. (○). 11 S protein: (●). 2 S protein.

Urea also caused the dissociation of the 11 S protein. At 1 M urea two peaks with S_{app} values of 11 S and 7 S were observed (figure 3A). Above this concentration, 3 peaks with values of 11 S, 7 S and 2 S were observed (figure 3B). The

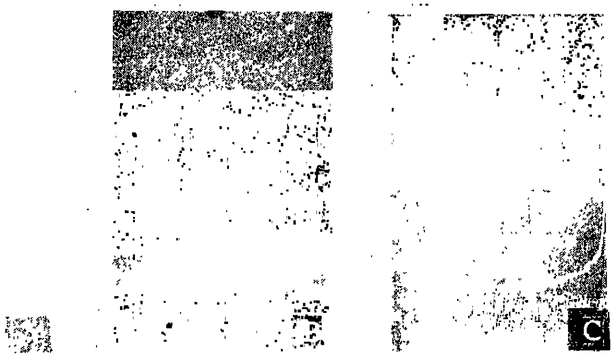

Figure 3. Sedimentation velocity pattern of 11 S protein in the presence of urea (0.025 M tris-glycine buffer, pH 8.3).
A. 11 S protein +1 M urea; B. 11 S protein +2 M urea; C. 11 S protein +6 M urea. (Sedimentation proceeds from left to right.)

proportion of the 11 S peak decreased continuously with increase in urea concentration and that of the 2 S peak increased (figure 4). The 7 S peak showed a different behaviour; its proportion increased gradually to a maximum of 35% at 2 M urea and then decreased at higher concentrations. At 6 M urea, neither the 11 S nor the 7 S peak was observed and 2 S peak alone was observed (figure 3C).

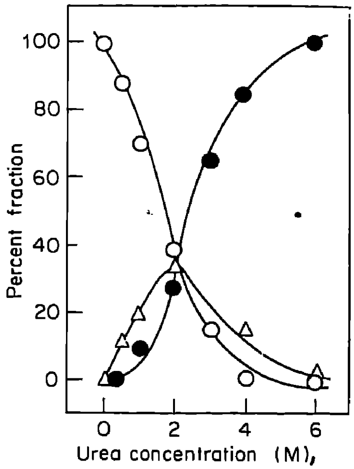

Figure 4. The proportion of protein fractions as a function of urea concentration. (O), 1 S protein; (Δ), 7 S protein; (●), 2 S protein.

GuHCl also caused the dissociation of the 11 S protein into 7 S and 2 S proteins. At 0.5 M GuHCl, two peaks, 11 S and 7 S, were observed (Figure 5A).. Above this concentration, 3 peaks—11 S, 7 S and 2 S were observed (figure 5 B). At 3 M

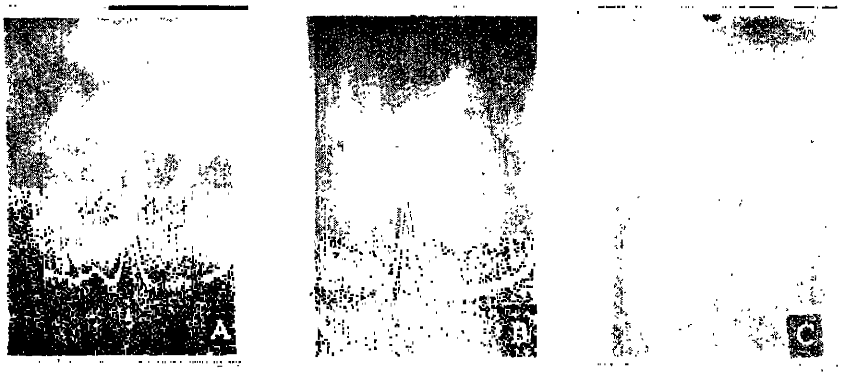

Figure 5. Sedimentation velocity pattern of 11 S protein in the presence of GuHCl (0.025 M tris glycine buffer, pH 8.3).

A. 11 S protein +0.5 M GuHCl; B. 11 S protein +1.0 M GuHCl; C. 11 S protein +3.0 M GuHCl. (Sedimentation proceeds from left to right).

GuHCl, 2 S peak alone was observed (figure 5C). The proportion of the 7 S peak increased to a maximum of ~25% (at 1.5 M GuHCl) and then decreased (figure 6).

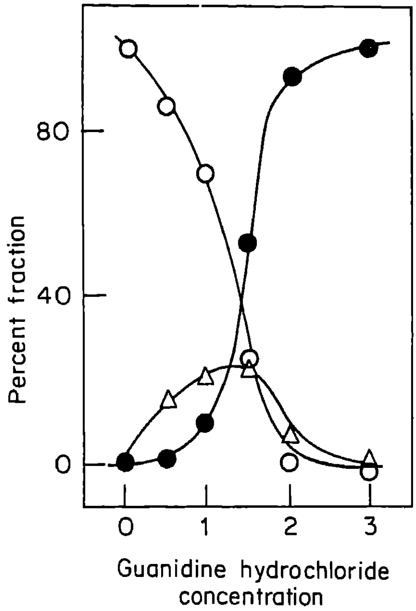

Figure 6. The proportion of protein fractions as a function of GuHCl concentration. (O), 11 S protein; (Δ), 7 S protein; (●), 2 S protein.

On SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (1969), the 11 S protein gave atleast 10 bands. However, in sedimentation velocity experiments, the 11 S protein in the presence of SDS gave only two peaks with S_{app} values of 11 S and 2 S. This apparent contradiction could be due to differences in the experimental conditions. The solutions for SDS-PAGE experiments contained β-mercaptoethanol whereas those for sedimentation velocity experiments did not; also the latter solutions had not been incubated at 37°C for 24 h in presence of SDS and thus dissociation may not have been complete. Further, the peaks observed in the sedimentation experiments may not represent homogeneous protein species and may be due to proteins with close mol. wt.

SDS directly dissociated the 11 S protein to the 2 S protein. On the other hand, with urea and GuHCl, dissociation appeared to occur through an intermediate 7 S protein. Schematically it can be written

$$11\ \text{S} \xrightarrow[\substack{\text{or GuHCl} \\ \text{(low concn.)}}]{\text{Urea}} 7\ \text{S} \xrightarrow[\substack{\text{GuHCl} \\ \text{(high concn.)}}]{\text{Urea or}} 2\ \text{S}$$

The dissociative effect of GuHCl was greater than that of urea as has been reported (Tanford, 1968). For this comparison, the weight-average sedimentation coefficient was calculated by following the movement of the second moment of the entire gradient curve according to the procedure of Goldberg (1953) and plotting this value as a function of denaturant concentration. In figure 7, such plots for urea and GuHCl are given. Sigmoidal curves were obtained and the mid-points of the transition curves were at 1.7 M for GuHCl and 2.5 M for urea. In the case of SDS, it occurred at a much lower concentration, namely 0.02 M (0.58%).

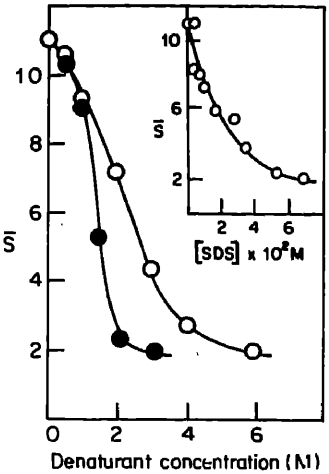

Figure 7. The weight average sedimentation coefficient, $\bar{S}$, as a function of denaturant concentration.
(O), Urea; (●), GuHCl.

The distinctive features of the dissociation of the 11 S protein by SDS were that (i) it occurred at low concentrations of SDS (compared to urea or GuHCl) and (ii) it converted directly to the 2 S protein.

The subunits of 11 S protein of the sunflower are likely to be held together by ionic linkages, hydrogen and hydrophobic bonds (Shetty and Narasinga Rao, 1978; Appu Rao and Narasinga Rao, 1979). SDS has both ionic and hydrophobic groups and it is also strongly bound to proteins (Steinhard and Reynolds, 1969; Reynolds *et al.*, 1970; Reynolds and Tanford, 1970). The strong dissociative action of SDS on the 11 S protein could be due to its ability to break both ionic linkages, and hydrophobic (and hydrogen) bonds (Jencks, 1969). This could be the reason for the greater dissociative effect of SDS than urea or GuHCl. Also the conformational stability of the 7 S protein could be low so that a powerful dissociating agent like SDS can dissociate it to 2 S, immediately after its formation.

Variable effects of SDS, urea or GuHCl on oliogomeric vegetable proteins have been reported. SDS dissociates α-globulin of sesame (*Sesamum indicum* L.), which is a 11 S protein, into 7 S, 4 S and 2 S proteins (Prakash and Nandi, 1976). On the other hand, urea causes association of the protein at low concentrations (1-3 M) of the denaturant followed by dissociation (Prakash and Nandi, 1977). In the case of GuHCl, a precipitate is observed at low concentrations and at 1.5 M GuHCl, 95% of the 11 S protein is dissociated into the 2 S protein. Gururaja Rao an Narasinga Rao (1979) have reported that SDS, upto 0.1% concentration, cause association of the 12 S protein of mustard (*Brassica juncea*) and at higher concer trations, dissociates it into 1.8 S protein. In urea solution (3-8 M), both aggrega tion and dissociation into 1.8 S protein were observed; however no aggregation wa observed in GuHCl solutions (Gururaj Rao, 1980). 12 S protein of rapesee (*Brassica campestris*) is dissociated into 1.8 S protein at all SDS concentration (Gururaj Rao, 1980). SDS dissociates the 11 S protein of guar seed (*Cyamopsi tetragonoloba*) into 2 S protein, whereas, urea or GuHCl dissociates it into 7 S and S proteins (Nath, 1980).

Effect of heat

Heating the 11 S protein in 0.025 M tris-glycine buffer of pH 8.3 in the range 30 90°C did not precipitate the protein. Only turbidity developed and this increase with increase in temperature. Turbidity, expressed quantitatively as $[(T_0-T)/T_0]\times$ 100, is plotted against the temperature of heating in figure 8. Upto 75°C there wa no turbidity. Above 80°C it increased sharply and reached a value of nearly 100 a

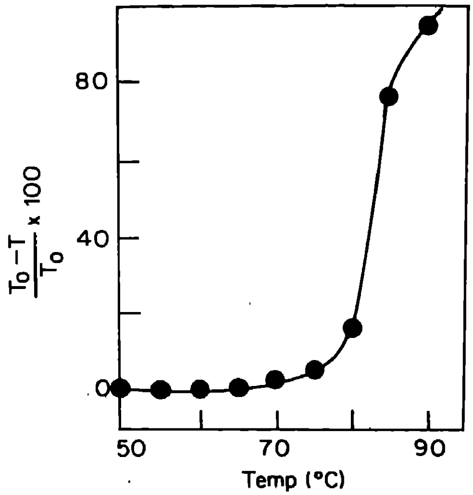

Figure 8. Turbidity of 11 S protein solution as a function of temperature of heating.

90°C. The temperature corresponding to a turbidity value of 50 was 83°C. Th indicates that increase in temp. causes development of turbidity.

The effect of heating interval on turbidity was also determined. Heating for min did not develop turbidity at any temperature. However, heating for 10 mi developed turbidity and the value $[(T_0-T)/T_0]\times 100$ did not increase when th solution was heated for longer intervals. The data given in figure 8 refer to 10 m heating.

The protein solution heated for 10 min at various temp was examined by PAG to determine the nature of changes that had occurred. The PAGE pattern of th

11 S protein consisted of a single band (figure 9). Heating at 80°C did not cause any change. The pattern of the protein heated at 85°C showed a diffuse fast moving band in addition to that of the 11 S protein. The sample heated at 90°C had a higher proportion of the fast-moving band.

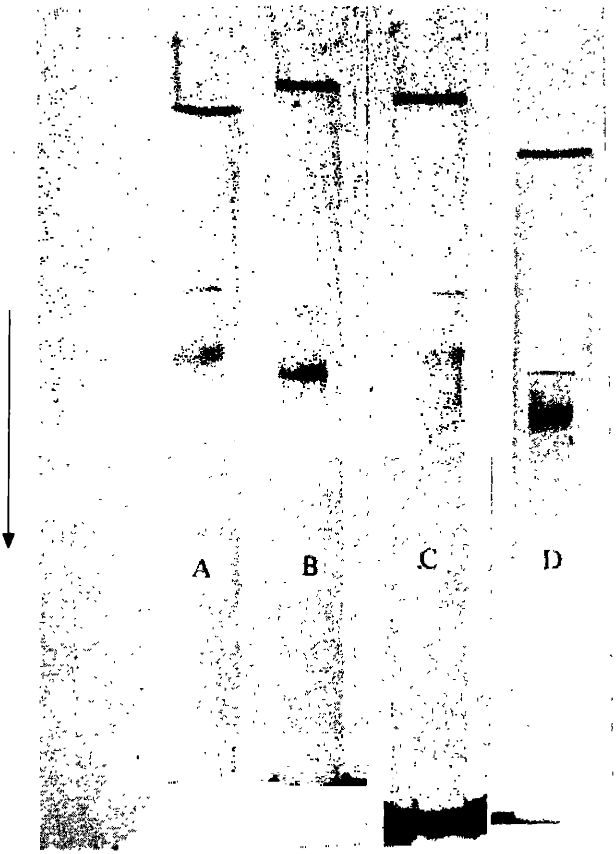

Figure 9. Polyacrylamide gel electrophoresis pattern of 11 S protein heated at different temperatures (0.02 M tris-glycine buffer, pH 8.3).

A. 11 S protein unheated: B. 11 S protein heated at 80°C for 10 min; C. 11 S protein heated at 90°C for 10 min; D. 11 S protein heated at 90°C for 20 min. ↓ Direction of migration.

When the protein solution was heated at 90°C for intervals longer than 10 min, the proportion of the fast-moving band increased and the sample heated for 20 min showed no band in the position of the 11 S protein. However, as stated earlier, there was no change in the turbidity value. Thus, heat also caused dissociation of the 11 S protein of sunflower.

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DNA Sequence organization in finger millet *(Eleusine coracana)**

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Abstract. Approximately 39 to 49% of the genome of finger millet consists of repetitive DNA sequences which intersperse with 18% of single copy DNA sequences of 1900 nucleotide pairs. Agarose gel filtration and electrophoresis experiments have yielded the sizes of interspersed repeated sequences as 4000-4200 nucleotide pairs and 150-200 nucleotide pairs. Approximately 20% of the repeated DNA sequences (4000-4200 nucleotide pairs) are involved in long range interspersion pattern, while 60% of the repeated DNA sequences (150-200 nucleotide pairs) are involved in short period interspersion pattern.

Based on the data available in literature and the results described here on DNA sequence organization in plants, it is proposed that plants with haploid DNA content of more than 2.5 pg exhibit mostly the short period interspersion pattern, while those with haploid DNA content of less than 2.5 pg show diverse patterns of genome organization.

Keywords. DNA sequence organization; repetitive DNA; millet species.

Introduction

DNA sequence organization refers to the arrangement of repeated and non-repeated DNA sequences in a genome. Such information is expected to throw light on several important questions such as gene regulation. To date, DNA sequence organization has been studied in a large number of animal and plant species (Nagl, 1978). Based on these data, it is now known that there is an interspersion of repeated and single copy DNA sequences and that there are two main patterns of DNA sequence interspersion viz., short period and long period. Most of the eukaryotic species studied so far have revealed the presence of short period interspersion pattern, whereas a few insects such as *Drosophila* exhibit the long range interspersion pattern (Manning *et al.,* 1975; Crain *et al.,* 1976). Plant genomes differ from those of animals in two important respects, firstly, they have a relatively greater proportion of repetitive DNA sequences; secondly, the lengths of the interspersed repeated sequences are highly variable in the different species.

We have recently described the genome characterization of three Gramineae species, namely, finger millet, pearl millet and rice, with rather low DNA content

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* NCL Communication No.: 2708

Abbreviations used: UV, ultra violet.

(Deshpande and Ranjekar, 1980). We have also carried out the DNA sequence organization studies in these species to determine whether they differ from the other well characterized Gramineae species, namely, rye (Smith and Flavell, 1977) and wheat (Flavell and Smith, 1976) in the mode of arrangement of repeated and single copy DNA sequences. In this paper, we report the data on DNA sequence organization in finger millet (*Eleusine coracana* Gaertn).

Materials and methods

All the chemicals used throughout the work were of Analytical Reagent (AR) grade or Guaranteed Reagent (GR) grade obtained from BDH, Sarabhai Chemicals and E. Merck. Certain chemicals such as RNAse, PIPES, Tris were obtained from Sigma Chemical Corporation, USA. λDNA, S_1 nuclease, EcoRI and DNA molecular weight markers were secured from Boehringer Mannehiem, West Germany.

Isolation of DNA

DNA of finger millet was isolated from shoots of eight day old plants as described earlier (Deshpande and Ranjekar, 1980).

DNA shearing and sizing

DNA fragments of an average length of 550 nucleotide pairs were obtained by sonication for three min using Biosonic III (Bronwill model, 250 W with 20 KHz) fitted with a half inch probe. Sonication with a ¼ inch probe for one min at 20 setting yielded DNA fragments of 1500 nucleotide pairs. Larger DNA fragments (4900 nucleotide pairs) were obtained by homogenizing the DNA solution in a Sorvall Omnimixer (Model No. 17106) for 6 min at 25,000 rpm. DNAs of fragment size in the range of 1000-3000 nucleotide pairs were obtained using a Virtis 60 homogenizer by varying the conditions of speed and time in the range of 29,500 to 58,000 rpm and 4 to 45 min respectively. The average size of the DNA fragments after shearing was determined by sedimentation through neutral sucrose gradients (5-25% in 0.15 M NaCl+0.015 M Trisodium citrate, pH 7.0) in a Beckman preparatory ultracentrifuge (McConkey, 1967; McEwen, 1967; Van der Schans, *et al.*, 1969).

Kinetics of DNA reassociation

The reassociation kinetics of finger millet DNA of all sizes were studied essentially by the method of Britten, *et al.* (1974). The separation and estimation of denatured and reassociated DNA fractions were carried out as described earlier (Ranjekar and Murthy, 1973; Ranjekar *et al.*, 1974).

Thermal denaturation

Thermal denaturation studies of native finger millet DNAs of all sizes and of the repetitive fractions were carried out in a Gilford Spectrophotometer 250 equipped with a thermoprogrammer (Model No. 2527), an analog multiplexer (Model No. 6046) and a reference compensator (Ranjekar *et al.*, 1976). In these studies, the data were used without any correction.

Single strand collapse was determined by melting unreassociated unique finger millet DNA in 0.12 M sodium phosphate buffer, pH 6.8. This value was observed to be 1-1.5%.

Digestion with S_1 nuclease

Native DNA of fragment size 5700 nucleotide pairs was used for S_1 nuclease digestion according to the procedure detailed earlier (Seshadri and Ranjekar, 1980).

Size fractionation of S_1 nuclease resistant reassociated duplexes

The size distribution of S_1 resistant duplexes was determined by gel filtration on an Agarose A_{50} (100-200 mesh size, Biorad Laboratories) column (92×1.5 cm), previously calibrated with calf thymus DNA of known duplex lengths. Approximately 70-100 μg of S_1 nuclease-resistant DNA duplexes, separated by hydroxyapatite chromatography, were loaded on the column and the elution was carried out with 0.12 M sodium phosphate buffer, pH 6.8 (Sachs and Painter, 1972; Wimpee and Rawson, 1979).

The fractions from this column corresponding to the peaks on the ultraviolet (UV) monitor were then analyzed by Agarose gel electrophoresis for the determination of size. Electrophoresis was carried out as described by Thomas and Davis (1975) with some modifications. λDNA, digested with EcoRI was used as a molecular weight marker in these experiments.

Results

Reassociation kinetics of 550 nucleotide pair long finger millet DNA

Figure 1 represents the reassociation kinetics of 550 nucleotide pair long DNA from finger millet. The solid line is the experimental curve, while the dashed line through the data points reveals the computer fit for second order kinetics using a nonlinear least square analysis method. A standard optimization subroutine STEPIT (written by J. P. Chandler and distributed by Quantum Chemistry Programme Exchange, Indiana Universitity, Bloomington, Indiana, USA) was used for this analysis keeping all the parameters to free float. It clearly indicates the presence of two kinetic components with a root mean square value of 0.0261. The fast reassociating component represents 49% of the genome and is repetitive. The latter 42% of the genome reassociates slowly with a rate of 0.00194 mol^{-1} s^{-1}. A part of the genome 8.5% fails to reassociate at Cot 10,000 mol×s/l. The lower dashed curves in figure 1 represent the predicted reassociation kinetics of the individual components if they existed alone.

Reassociation kinetics of DNAs of different fragment lengths

The reassociation kinetics of DNAs from finger millet, of four different fragment lengths in the range of 550-5700 nucleotide pairs were studied (figure 2). As no labelled DNAs were used throughout the work, the reassociation experiments were carried out in the Cot range of 10^{-2} to 2.5×10 mol. s.l^{-1}. By Cot 2.5×10 mol. s.l^{-1}, almost all repetitive DNA sequences and very few single copy sequences are assumed to reassociate (Deshpande and Ranjekar, 1980). The extent of hydroxy-

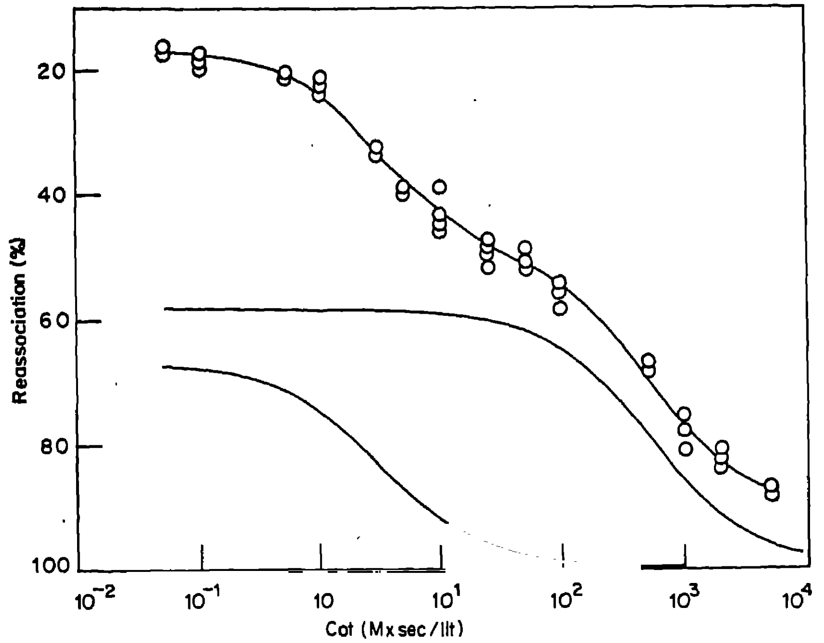


Figure 1. Reassociation kinetics of 550 nucleotide pair long finger millet DNA by hydroxyapatite fractionation.

The line through the data points represents the least square fit for two component system allowing all the parameters to free float (root-mean-square, 0.026). The lower dashed curves represent the reassociation kinetics of pure components. The DNA solutions (25-200 μg/ml) in 0.12 M sodium phosphate buffer (pH 6.8) were first denatured at 100°C and incubated at 62°C to obtain a desired Cot value. The unreassociated and reassociated DNA fractions were isolated at 0.12 M and 0.4 M sodium phosphate buffer (pH 6.8) respectively.

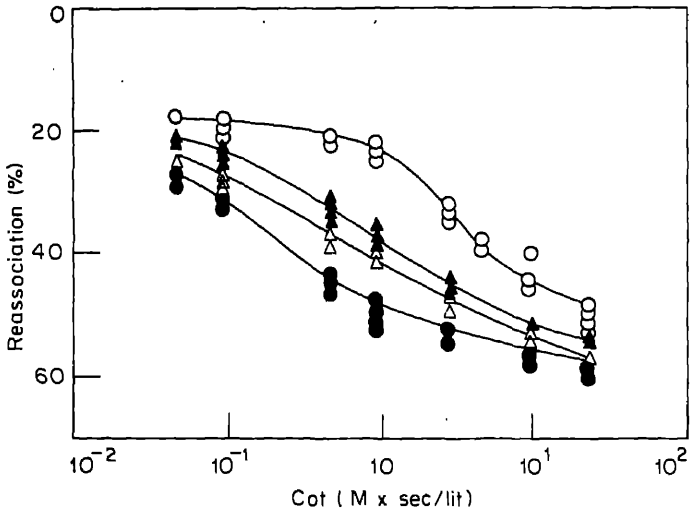


Figure 2. Hydroxyapatite reassociation kinetics of finger millet DNA at fragment lengths 550 nucleotide pairs (O), 1500 nucleotide pairs (— ▲ —), 4900 nucleotide pairs (Δ), and 5700 nucleotide pairs (●).

apatite binding increases from 18% to 28% and from 49% to 58% at Cot 0.5 and 25 mol. s.l^{-1}, respectively as the fragment length increases from 550 nucleotide pairs to 5700 nucleotide pairs. The increase in the binding of reassociated duplexes to hydroxyapatite can be either due to interspersion of repeated and single copy sequences or due to the effect of DNA fragment length alone. If there is no interspersion of different kinetic components, then the reassociation rate will solely be affected by the fragment length and the observed rate constants of repetitive DNA fractions will only vary as a function of the square root of the ratio of the fragment lengths of the two fractions being compared as represented by the following formula (Wetmur and Davidson, 1968):

$$K_1/K_2 = (L_1/L_2)^{0.5}$$

where L_1 = shorter fragment size as number of nucleotide pairs
L_2 = longer fragment size as number of nucleotide pairs
K_1 = rate constant for reassociation of shorter (L_1) fragments
K_2 = rate constant for reassociation of longer (L_2) fragments

However, DNA fragments containing both single copy and repetitive sequences will have rate constants greater than those predicted as above. Table 1 gives the observed and the predicted rate constants for fragments of different lengths. The ratios, of observed rates to predicted rates vary in the range of 3.0 to 6.0 suggesting the presence of single copy DNA sequences in an unreassociated form contiguous to repetitive duplexes. Thus, it appears that about 58% of the total genome of finger millet (5700 nucleotide pairs) consists of interspersed sequences.

Table 1. Comparison of the experimental and the predicted rate constants (K) of the repeated DNA fractions of different fragment lengths.

Fragment length nucleotide pairs	Cot 1/2 observed[a] mol. S. l^{-1}	Observed K[b] l. mol^{-1} S^{-1}	Predicted[c] l. mol^{-1} S^{-1}	$\frac{\text{K observed}}{\text{K. predicted}}$
550	1.4×10^{0}	0.714	—	—
1500	2.0×10^{-1}	5.000	1.179	4.440
4900	1.25×10^{-1}	8.000	2.1319	3.7525
5700	7.5×10^{-2}	13.00	2.2994	5.784

[a] Calculated from the reassociation curves upto Cot 25 in figure 2.

[b] $K = \frac{1}{\text{Cot 1/2 observed}}$

[c] Calculated using $\frac{K1}{K2} = \frac{L1}{L2}^{0.5}$

where K1 and K2 are the rate constant for the reassociation of short (L1) and long (L2) fragments, respectively.

Hyperchromicity studies of reassociated repetitive DNA fragments

As observed in figure 3, the melting profiles of Cot 25 fractions of different fragment lengths of finger millet DNA are smooth. There is an increase in the

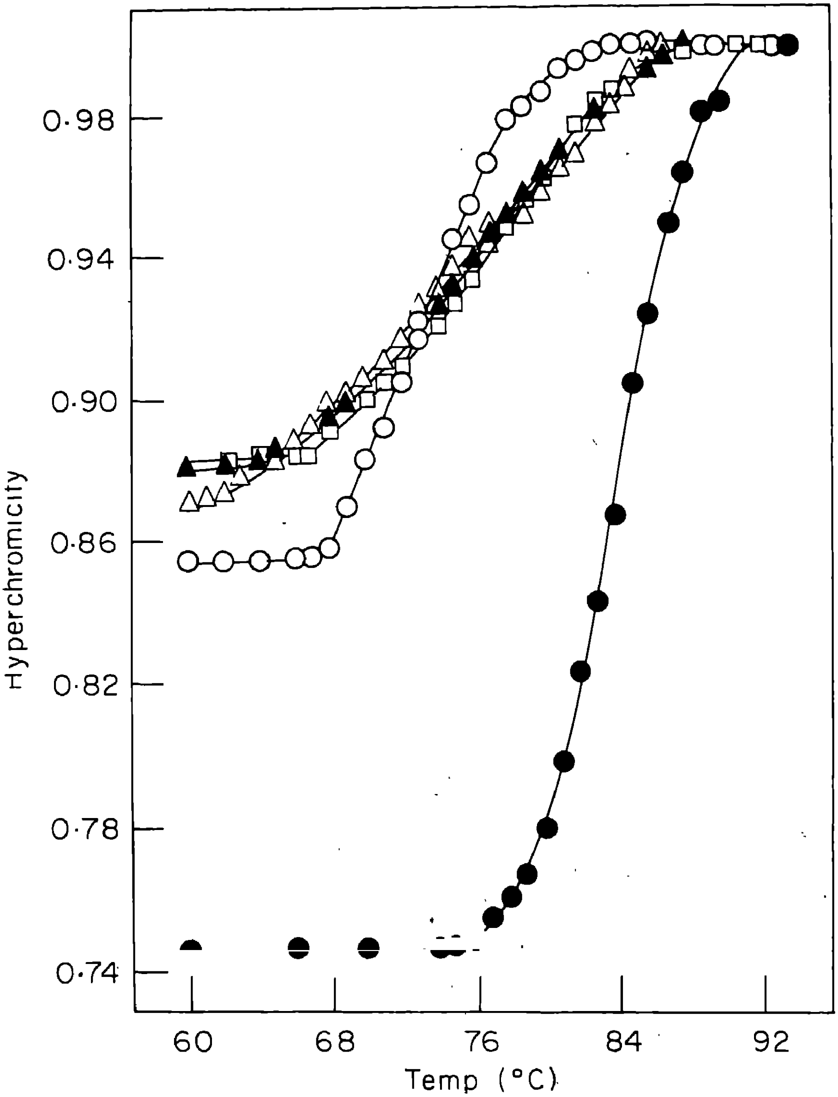


Figure 3. Thermal denaturation profiles of finger millet Cot 25 DNA of various fragment lengths, compared to those of native, 550 nucleotide pair long DNA.

The reassociated duplexes at Cot 25 were separated on hydroxyapatite. These fractions were dialyzed against 0.12 sodium phosphate buffer (pH 6.8) and were melted in a Gilford 250 spectrophotometer by increasing the temperature at a rate of 1°C/min. the graphs of relative absorbance (A260(TI)/A260(98°C) *vs* temperature were plotted where A260 (TI) is the absorbance at the corresponding temperature. (O) 550 nucleotide pairs; (Δ), 1500 nucleotide pairs; (▲), 4900 nucleotide pairs (□), 5700 nucleotide pairs; (●), native, 550 nucleotide pair long DNA. Each point in these graphs represents an average of atleast five experiments.

melting temperature of the reassociated duplexes (1.5°-4.0°C) while the hyperchromicity decreases with increasing fragment length (14% to 11.6%). Such decrease in the hyperchromicity of a repetitive DNA fraction with increase in the size of the DNA fragment is attributed to the increasing presence of single strand regions in the reassociated fragments. These single stranded regions are, most likely, unique sequences which remain unreassociated at Cot 25 and contribute very little (about 1-1.5%) to DNA hyperchromicity. These data, thus provide an additional evidence for the existence of repetitive and nonrepetitive DNA sequences on the same strand in finger millet genome.

Using hyperchromicity values, we calculate that about 50% of the 550 nucleotide pair long DNA is base-paired while only 39% of the 5700 nucleotide long fragments is base-paired. The later value is slightly lower as compared to the repetitive DNA content revealed from the reassociation curve of the 550 nucleotide pair long DNA (figure 1). The average size of the interspersed repetitive DNA sequences which reassociate within the 5700 nucleotide pair fragment is computed to be 2223 nucleotide pairs (table 2).

Table 2. Melting analysis of Cot 25 DNA isolated from DNAs of different fragment lengths.

DNA fragment size (nucleotide pairs)	550	1500	4900	5700	Native sonicated DNA (550 nucleotide pairs)
Fraction bound to HA at Cot 25[a]	0.49	0.535	0.575	0.58	—
Hyperchromicity[b]	0.142	0.13	0.118	0.114	0.27
Tm °C[c]	73.0	75.0	76.5	77.0	—
Duplex content (D)[d]	0.498	0.459	0.403	0.390	—
Average duplex length[e]	273	676	1979	2223	—
Duplex content from S_1 nuclease[f]	—	—	—	0.41	—

[a] Obtained from figure 2.

[b] Obtained from figure 3. Hyperchromicity (H) was calculated using the formula

$$H = \frac{\text{A260 (98°C)} - \text{A260 (60°C)}}{\text{A260 (98°C)}}$$

(Zimmerman and Goldberg, 1977; Wimpee and Rawson 1977)

[c] Obtained from figure 3.

[d] The average duplex content (D) of bound fragment was estimated using the formula:

$$D = \frac{\text{H} - \text{single strand collapse}}{\text{H (native sonicated DNA)} - \text{single strand collapse}}$$

The hyperchromicity of the denatured single copy DNA was determined in order to obtain a value for single strand collapse and was of the order of 1.5% (Graham *et al.*, 1974)

[e] The average length of duplex region is the product of the duplex content (D) and the fragment length of DNA.

[f] Duplex content of S_1 nuclease treated repetitive DNA fragment was obtained as:

$$\frac{\mu\text{g of S1 nuclease resistant repetitive duplexes bound to hydroxyapatite column}}{\mu\text{g of S1 nuclease treated total DNA loaded on hydroxyapatite column}}$$

S_1 nuclease studies of reassociated repetitive DNA fragments

An independent approach to determine the presence of single strand regions in th reassociated fractions is to carry out S_1 nuclease experiments. The hyperchrom city of Cot 25 fraction isolated from 5700 nucleotide pair DNA is 11.6% while tha of the S_1 nuclease resistant Cot 25 DNA is 21.5% (figure 4). This further indicate that the single strand regions in the DNA fraction are mostly digested by S nuclease.

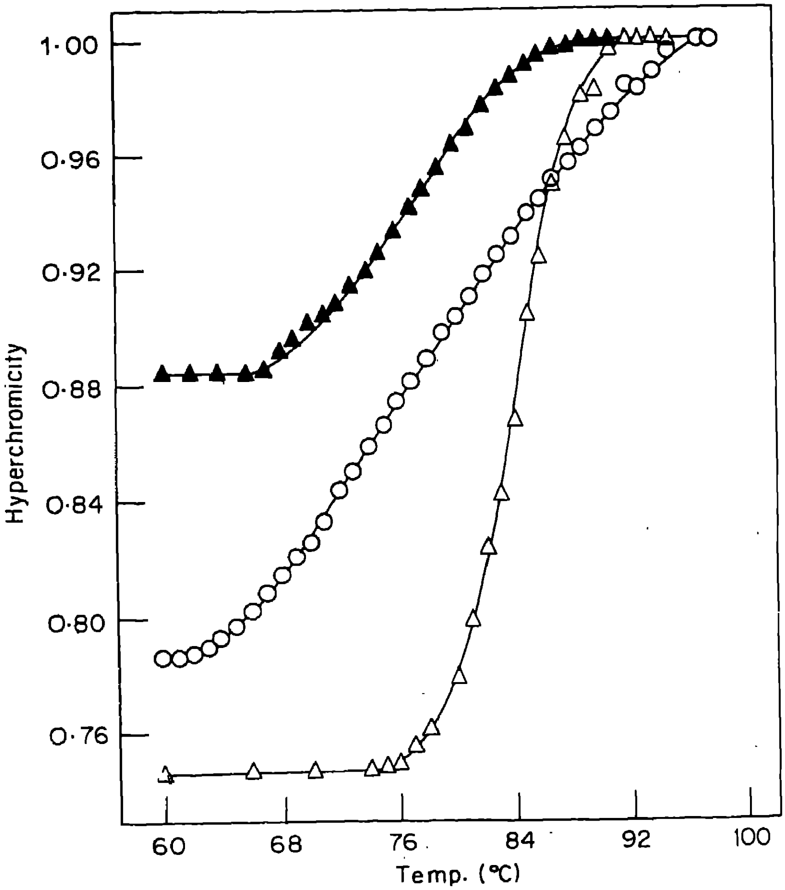


Figure 4. Thermal denaturation profiles of Cot 25 finger millet DNA before and after S_1 nuclease digestion along with native, 550 nucleotide pair long DNA.

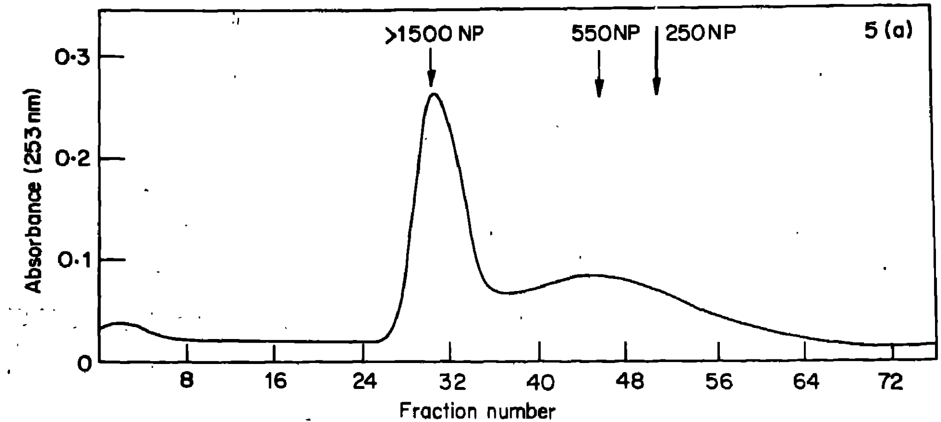
Native DNA of 5700 nucleotide pairs was dialysed against 0.18 M NaCl/0.006 M PIPES buffer, pH 6.8. The DNA samples were denatured at 100°C for 10 min and then reassociated to specific Cot values. After the required incubation, the DNA solution containing mixture of reassociated and unreassociated nucleotide fragments was incubated with S_1 nuclease for 1 h at 37°C to digest the single stranded fragments. The S_1 nuclease resistant duplexes were isolated by hydroxyapatite column chromatography and were dialyzed against 0.12 M sodium phosphate buffer (pH 6.8). The hyperchromicity curves were plotted as described in figure 3. Before S_1 nuclease treatment, (▲); after S_1 nuclease treatment, (○) and native 550 nucleotide pairs long DNA (Δ). In these experiments, each point represents an average of three different experiments using three different DNA preparations.

S_1 nuclease experiments can also be used to provide an independent estimate of the fraction of nucleotides which are in duplex form in the hydroxyapatite binding DNA fraction. Approximately 41% of 5700 nucleotide pair long DNA fraction, which is capable of binding to hydroxyapatite at Cot 25 is S_1 nuclease resistant and hence is in duplex form. This estimate compares well with the estimated value of 39% obtained from hydroxyapatite measurements. Since we assume that all the repetitive DNA sequences reassociate by Cot 25, the fraction of fragments which could bind to hydroxyapatite (5700 nucleotide pairs) and which are in duplex form, most probably represent the per cent of repetitive sequences. The proportion of single copy DNA will, therefore, be 59-61%. This value is in good agreement with that obtained in the experiment on reassociation kinetics of 550 nucleotide pair long DNA (figure 1).

Agarose gel column chromatography and Agarose gel electrophoresis of S_1 nuclease-resistant repetitive DNA

A direct estimate of the size is distribution of repetitive DNA sequence was obtained by analyzing S_1 nuclease resistant repetitive duplexes using Agarose column chromatography.

Since the exclusion limit of the Agarose A_{50} column is 1500 nucleotide pairs, all the DNA sequences having a length greater than 1500 nucleotide pairs are excluded from the column. The first peak in figure 5b indicates these sequences. By comparison with the column chromatographic profile of calf thymus DNA of known duplex lengths, the second fraction in figure 5b appears to consist of DNA sequences of length 150-200 nucleotide pairs. The proportion of the excluded DNA fraction (greater than 1500 nucleotide pairs) ranges from 18% to 23%; while that of the second fraction varies from 57% to 64%. The remaining 18% to 20% of the repetitive duplexes have a broad size distribution in the range of 500 to 1000 nucleotide pairs. The precise lengths of these fractions were then determined by Agarose gel electrophoresis (figure 6). From the mobility of the excluded fraction in gel electrophoresis, the molecular weight was estimated to be 4000-4200 nucleotide pairs. The low molecular weight fraction of repetitive DNA shows a very high mobility corresponding to a length of less than 500 nucleotide pairs. Considering Agarose column chromatography and electrophoresis results together, the length of the low molecular weight DNA fraction of finger millet is determined to be 150-200 nucleotide pairs.



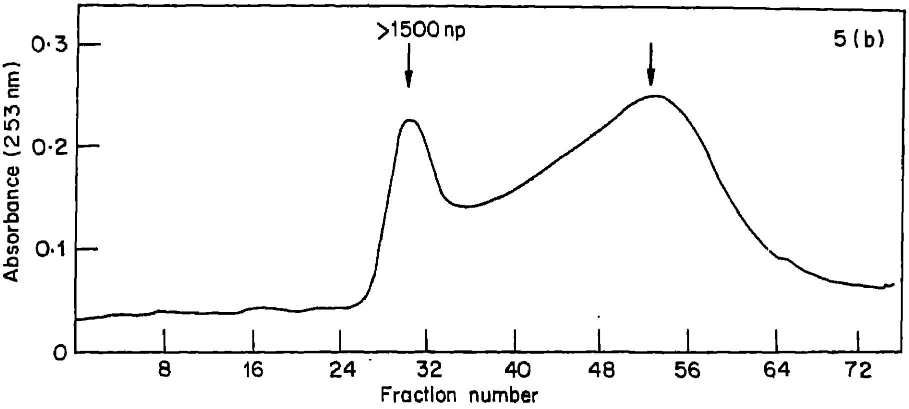
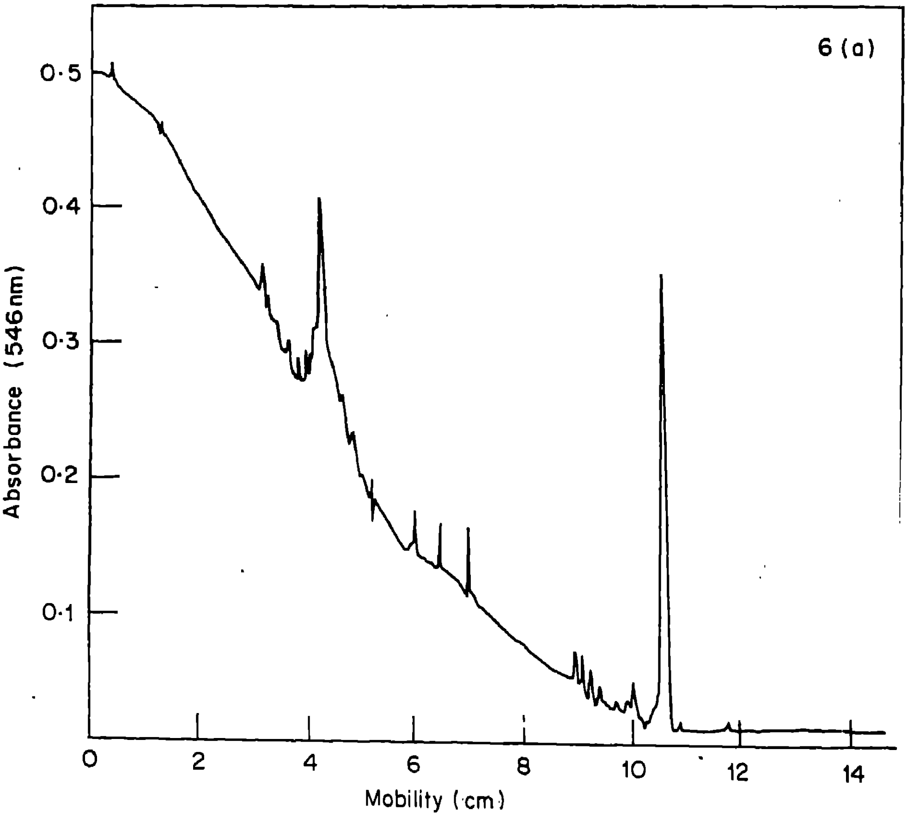


Figure 5. Agarose A50 column chromatographic profile of (a) calf thymus DNA of known duplex lengths (b) S_1 nuclease resistant Cot 25 finger millet DNA.

The size distribution of S_1 resistant duplexes was determined by gel filtration on an Agarose A50 (Bio-Rad) column using 0.12 sodium phosphate buffer (pH 6.8) and the scans were obtained by monitoring the effluent with the help of an LKB Univord at 253 nm.



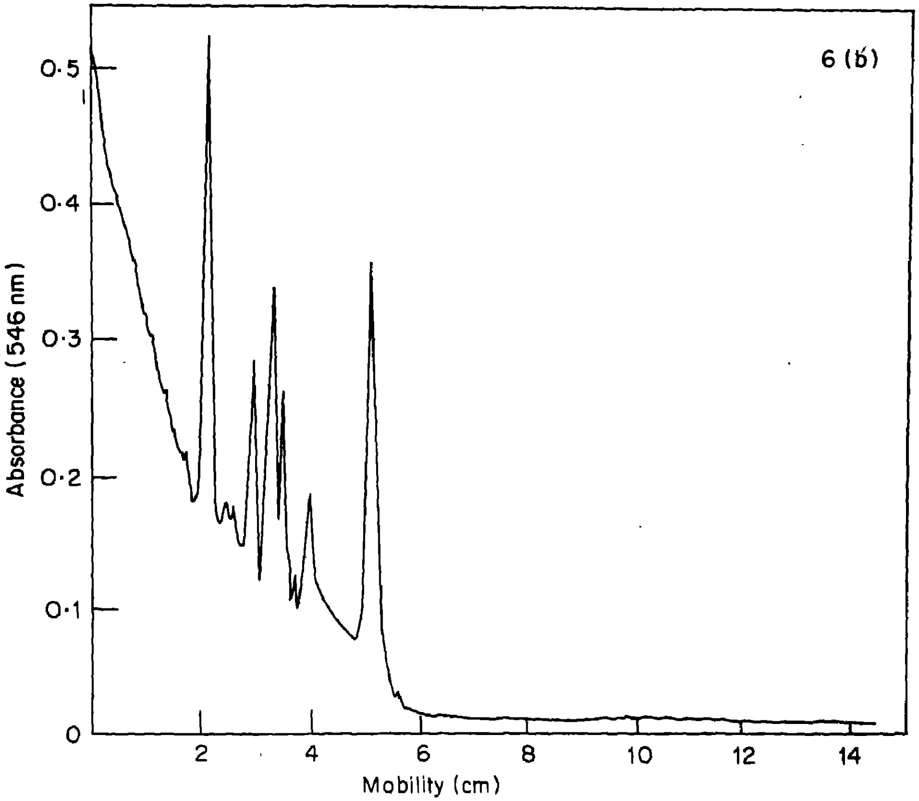


Figure 6. Agarose gel electrophoresis of S_1 resistant repetitive duplexes.

The fractions eluted from Agarose column were loaded on Agarose gels (0.6 cm×15 cm) and electrophoresis was carried out at 50 V for 5 h at room temperature. The profiles were obtained by scanning the gels at 546 nm in a Gilford 250 spectrophotometer equipped with a gel scanner. (a) Finger millet repetitive duplexes. (b) λ DNA digest with EcoRI as a DNA molecular weight marker. This electrophoretic scan compares very well with that of Thomas and Davis (1975) who have used ethidium bromide to stain the DNA bands and have taken the photographs in UV fluorescence.

Spacing of repetitive sequence elements

The length of the interspersed single copy sequences can be estimated from a curve relating the fraction of DNA fragments binding to hydroxyapatite and the fragment length. From figure 7, it is observed that two slopes are present and the change in the slope occurs at a fragment length of approximately 1800-2000 nucleotide pairs. Upto 1900 nucleotide pairs, there is a linear relationship between fragment length and per cent binding to hydroxyapatite and thereafter the increase in the binding is more gradual. Thus the length of the single copy DNA of finger millet DNA is in the range of 1800-2000 nucleotide pairs.

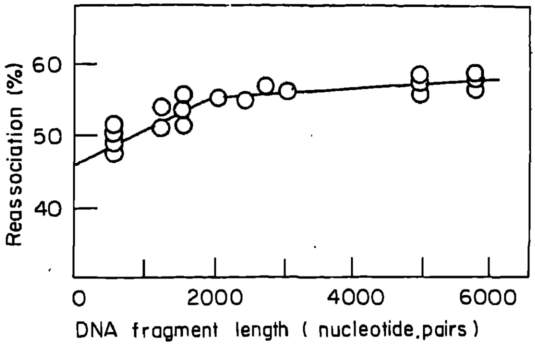


Figure 7. The percentage binding of finger millet DNA to hydroxyapatite at Cot 25 as a function of DNA fragment length. DNAs were sheared to various fragment lengths and adjusted to 0.12 M sodium phosphate buffer (pH 6.8). The percentage binding was calculated as described in figure 1.

An estimate of the percentage of repetitive DNA content can be obtained b extrapolating the curve of greater slope to a fragment length of zero. Th intercept reveals the percentage of repetitive DNA as 46% which is in clo agreement with the values obtained from reassociation kinetics, hyperchromici and S_1 nuclease measurements (table 3).

Table 3. Duplex content (D) of repetitive DNA fractions of finger millet from various independent methods

Method	Duplex content
Hyperchromicity measurements (table 2)	0.39
S_1 nuclease resistance measurements (table 2)	0.41
Reassociation (%) vs fragment length (figure 7)	0.46
Reassociation curve of 550 nucleotide pairs (figure 1)	0.49

Discussion

We have employed the most commonly used approach for studying the DN sequence organization for the analysis of the genome of finger millet. We have n been able to obtain labelled DNA of sufficiently high specific activity and henc have not used labelled tracer DNA. Vorobev and Kosjuk (1974) and Baldari ar Amaldi (1976) have clearly shown that their results obtained from sea urchin ar *Xenopus*, respectively, using unlabelled DNAs are in good agreement with th results in sea urchin (Graham *et al.*, 1974) and *Xenopus* (Davidson *et al.*, 197 where labelled DNA was used. In plants, Wimpee and Rawson (1979) have use unlabelled DNA for studying DNA reassociation kinetics in pearl millet. Sinc only unlabelled DNA was used in our experiments, we have been unable estimate the zero time binding fraction and to apply the corresponding correctic to the reassociation data.

From our data, it is clear that 58% of the total genome of finger millet consists of interspersed repetitive and nonrepetitive DNA sequences. About 18% of the single copy DNA sequences (1900 nucleotide pairs) are interspersed with 20% of 4000-4200 nucleotide pairs, 60% of 150-200 nucleotide pairs and 20% of intermediate size repetitive DNA sequences.

The information of DNA sequence organization in finger millet provides an additional strong evidence that plant genomes exhibit a great diversity in the length of the interspersed repeated DNA sequences. In fact, finger millet is the first plant genome where the lengths of the interspersed repeated DNA sequences are as large as that seen in pearl millet (Wimpee and Rawson, 1979) and smaller than those exhibiting typical short period interspersion pattern as in the case of wheat (Flavell and Smith, 1976), rye (Smith and Flavell, 1977), Soybean (Goldberg, 1978 and Gurley *et al.*, 1979), tobacco (Zimmerman and Goldberg 1977) and pea (Murray *et al.*, 1978). Thus the DNA sequence interspersion pattern in finger millet is different from that in other plants studied so far.

From the available data on plant species, a relationship can be postulated between the DNA content and the DNA sequence organization pattern. Plant species with a haploid genome size of more than 2.5 pg exhibit mostly the short period interspersion pattern which is typical of animal genomes; examples are wheat (17.3 pg), rye (7.9 pg), tobacco (3.9 pg), soybean (6.5 pg per cell) and pea (4.9-5.2 pg). On the other hand, plant species with a haploid DNA content of less than 2.5 pg show a diverse nature of genome organization. This is evident in cotton (0.795 pg per cell, Walbot and Dure, 1976), pearl millet (2.5 pg, Wimpee and Rawson, 1979), mung bean (0.5 pg, Murray *et al.*, 1979) and french bean (1.8 pg, Seshadri and Ranjekar, 1980). In these species, the length of the interspersed repeated sequences varies in the range of 1200-5000 nucleotide pairs. Finger millet falls under this category. In view of its wide occurrence, short period interspersion patten in eukaryotic genomes in assumed to be preferred to long period interspersion pattern during the course of evolution. Further, Britten and Davidson, (1969), and Davidson and Britten (1979) have proposed a model which is based on the significance of short period interspersion pattern in the coordinate regulation of transcription. However, considering the recent data on plant genomes, where interspersion patterns are not typically short period, it appears that the above model may have to be either altered or modified.

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Genetic and molecular events in transformation of *Haemophilus influenzae* with plasmid RSF 0885 carrying cloned segments of chromosomal DNA

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Abstract. In *Haemophilus influenzae*, genetic transformation for a plasmid marker is significantly increased when recombinant plasmid RSF 0885 DNA carrying chromosomal DNA segments is used instead of the plasmid DNA alone. Chromosomal DNA by itself, added even a few minutes after the addition of plasmid DNA to competent cells, stopped further uptake of the plasmid DNA. These observations are consistent with the idea that plasmid RSF 0885 contains a 'degenerate' version of the required eleven base-pair 'uptake sequence' in *Haemophilus*. The transformation activity of the recombinant plasmid DNA is recoverable after its entry into cells, although the specific biological activity of the re-isolated plasmid DNA is less than that of the parental recombinant plasmid DNA. The *rec 1* gene function of the host is necessary for obtaining higher transformation frequencies with recombinant DNA from five different clones. The reduced transformation frequencies seen in *rec* 1^- strain is not all due to a permanent damage to the donor DNA since the recovered recombinant plasmid DNA from such cells can increase the transformation efficiency on *rec* 1^+ strain.

Keywords. *Haemophilus influenzae;* Plasmid transformation; recombinant DNA; uptake sequences; *rec* gene requirement.

Introduction

Competent cells of *Haemophilus influenzae* take up efficiently only the DNA that contains a specific eleven base-pair sequence, about 600 copies of which are distributed through the *Haemophilus* genome (Sisco and Smith, 1979). Plasmid RSF 0885 originally isolated from *H. influenzae b* carrying ampicillin resistance (amp^r) marker, transforms competent cells of *H. influenzae* Rd with a frequency lower than 0.01% (Notani *et al.*, unpublished observations). These results suggest that either the required "uptake sequence" may be absent or that a 'degenerate' version of the sequence with a much lower affinity for the receptor protein(s) may be present. It is also possible that low transformation with RSF 0885 may be due to the events occurring subsequent to uptake. However, transformation frequencies are significantly increased by the plasmid DNA carrying a library of sequences of chromosomal DNA. The present paper describes the genetic and molecular events in transformation with the plasmid DNA carrying chromosomal DNA inserts.

Materials and methods

Bacterial strains

Haemophilus influenzae Rd is a wild-type strain from Dr. J. Setlow's collection. *r* 1 strain was also from her collection and is a recombination-deficient strai Plasmid RSF 0885 contained originally in a *Haemophilus* strain obtained fro Stanley Falkow was transferred to *H. influenzae* Rd strain in J. Setlow's laborato (BNL). Plasmid-bearing strains pD2, pD4, pD5, pD7 and pD9 were generated l selecting *amp*^{r} colonies by transformation with RSF 0885 DNA to which segmer of chromosomal DNA had been spliced.

Preparation of recombinant DNA

The detailed method will be described elsewhere. Briefly, purified plasmid DN and chromosomal DNA (Marmur, 1961) were digested with *Pvu II* and joined wi T4 ligase (New England Biolabs). A sample of the primary recombinant DNA w generously provided by Dr D. McCarthy.

Preparation and isolation of unlabelled and labelled plasmid DNA

Cleared lysates were prepared by minor modifications in the methods described Hirt (1967) as described below. Bacterial cultures were grown to stationary pha washed and resuspended in standard saline citrate (0.15 M NaCl and 0.015 M sodiu citrate) and lysed with 1% sodium dodecyl sulphate (SDS) (final concentration) 37°C for a minimum period of 30 min. NaCl (1 M final concentration) was gent mixed with the lysate. Lysates were stored at 4°C for at least 8 h. The lysates we then centrifuged at 20,000 *g* (rotor SS 34) in a Sorvall RC 2B centrifuge for 30 mi The supernatant (cleared lysate) contained bulk of the plasmid DNA and less th 5% of the chromosomal DNA. Lysates were treated with RNase and deproteiniz with chloroform: amyl alcohol (Marmur, 1961). The plasmid DNA was purifi from this lysate by ethidium bromide-cesium chloride equilibrium density-gradie centrifugation (Radloff *et al.*, 1967).

[^{3}H]-Labelled plasmic DNA was prepared by growing 8 ml cells in brain-hea infusion broth containing [^{3}H]-thymidine (33 μCi/ml) (Sp. activity 52 Ci/mm NEN). Cleared lysate (0.4 ml) was prepared as described above. An aliquot (0 ml) was layered on 5 to 20% sucrose (in SSC) gradients and centrifuged at ~75,00 for 2 to 2.5 h in a Beckman ultracentrifuge. On these gradients chromosomal DN sediments faster than the peak plasmid DNA. The amount of chromosomal DN which was contaminating peak plasmid DNA fractions was estimated to be le than a ng/ml. A part of the peak fractions was used for the assay of radioactivi and biological activity and was also used in transformation experiments.

Size determination of recombinant plasmid (pD7) DNA fragments

The recombinant 15 μl (pD7) plasmid DNA (~1μg) was cut with Pvu II (2.5 unit or Eco RI (10 units) for 1 h at 37°C and the digests were electrophoresed with Hpa digested T7 DNA standards (kindly provided by Alan Rosenberg), through 1 agarose gels as described by McDonnel *et al.* (1977).

Transformation

Transformation was done according to the MIV method (Steinhart and Herriott, 1968). For the 'fate' studies of input plasmid DNA, a 12 ml competent cell culture was exposed to DNA and 4 ml fractions were taken for each time point (in general, 0, 30 and 60 min). Cleared lysates were prepared from these cells as described above and the DNA from these lysates was used in a second transformation to assay for biological activity and radioactivity. In some cases, these assays were carried out on samples fractionated through sucrose density gradients.

Results

Inhibition of RSF 0885 DNA uptake by chromosomal DNA

At 1, 5, 10 or 20 min after mixing competent cells, with RSF 0885 DNA, excess of chromosomal DNA containing novobioin resistance marker was added. It was observed that even the subsequent addition of chromosomal DNA stopped further uptake/transformation of the plasmid marker (figure 1). This is consistent with our other observation (to be published elsewhere) that the kinetics of uptake of plasmid DNA are slower than that of chromosomal DNA and that chromosomal DNA effectively competes with plasmid DNA.

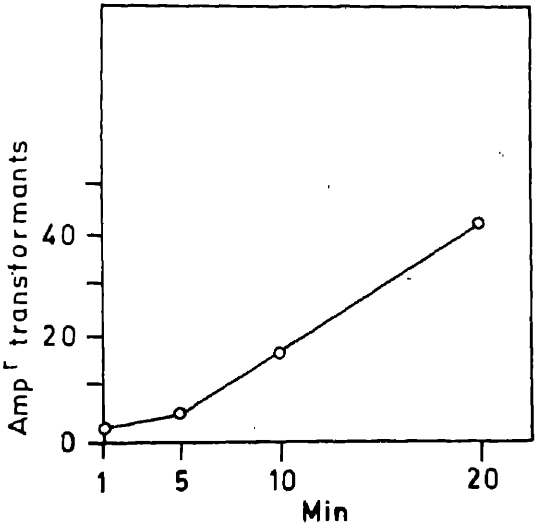

Figure 1. Effect of addition of excess of chromosomal DNA on transformation of *H. influenzae* by RSF 0885 DNA.

Transformation with plasmids carrying chromosomal DNA fragments

Transformation frequency with plasmid RSF 0885 is less than 0.01%. However, using recombinant plasmid DNA containing chromosomal DNA inserts yielded a burst of transformation. Ampicillin-resistant colonies picked from the cells that had been transformed with the primary recombinant DNA molecules were characterized further. Five of these were grown and cleared-lysates were prepared of the plasmid DNA. DNAs of the five isolates designated pD2, pD4 pD5, pD7 and pD9 were elctrophoresed through 1% agarose gels. All the isolates showed plasmids of varying sizes, with pD7 moving the slowest and therefore presumably had the largest insert(s) (data not shown). Transformation frequencies for *amp*^{r} marker with the five plasmids are given in table 1. These frequencies are higher by two to three orders of magnitude than that obtained with RSF0885 alone

Table 1. Transformation of wild type *H. influenzae* with recombinant plasmids carrying chromosomal DNA segments.

Plasmid DNA	*amp*^{r} transformants $(\times 10^6)$	Frequency of transformation (%)
pD2	3.1	0.6
pD4	7.5	1.5
pD5	4.3	0.9
pD7	5.2	1.0
pD9	4.2	0.8

Effect of DNA concentration on the number of transformations

Figure 2 shows the dose-response curve for pD7 DNA. Transformation increase linearly and then begins to saturate at less than 0.12 µg of plasmid DNA.

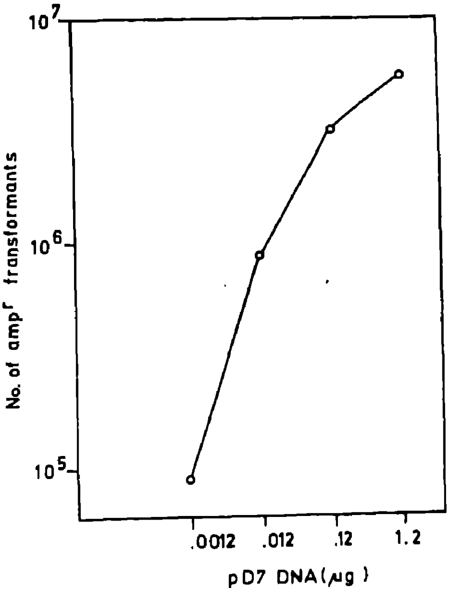

Figure 2. Effect of pD7 DNA concentration on plasmid transformation.

Determination of the size of pD7 DNA

When pD7 was cut with Eco RI, four fragments were obtained. Since, RSF 0885 is not cleaved by Eco RI, all the four cuts should have occurred in the chromosomal DNA. Molecular weight of these fragments was determined by gel electrophoresis of Eco RI digests of pD7 along with HpaI-cut T7 DNA standards. Molecular weight of pD7 was estimated to be about 12×10^6 (data not shown). Pvu II generated about 10 fragments which was surprising because the original splicing was done with Pvu II. It is possible that the original chromosomal DNA was either partially digested or that a number of independent segments were spliced or was rearranged.

Requirement for rec 1 gene expression for transformation with plasmids carrying chromosomal inserts

Following an observation of D. McCarthy (unpublished data) and of Setlow *et al.* (to be published elsewhere), it was observed that all the five plasmids under consideration here also require *rec* 1 gene expression for peak transformation frequencies (table 2). The reduction in the frequencies is almost three to four orders of magnitude. Reduction in transformation with chromosomal DNA or pD5 DNA in *rec* 1 is comparable (table 3).

Table 2. Transformation of *Rec*$^-$ 1 strain with recombinant plasmids carrying chromosomal DNA segments.

Recipient cells	plasmid DNA	*amp*^{r} transformants ($\times 10^2$)	Frequency (%)
nov rec 1	pD2	2	Less than 0.001
nov rec 1	pD4	33	
nov rec 1	pD5	35	
rec 1	pD7	6	
rec 1	pD9	15	

Table 3. Transformation with pD5 (*amp*^{r}) or chromosomal (*str*^{r}) DNA of wild type or *rec*$_1^-$ strain of *H. influenzae.*

Recipient cells	Donor DNA cells	No. of transformants
Wild type;	*str*^{r}	291×10^4
rec$_1$	*str*^{r}	0×10^1, 0×10^2
Wild type	*amp*^{r}	240×10^4
rec$_1$	*amp*^{r}	0×10^1, 0×10^1

Fractionation of $[^3H]$*-labelled pD7 DNA and efficiency of transformation*

A cleared-lysate of $[^3H]$-labelled pD7 was fractionated through 5-20% sucrose gradients. Since the resident chromosomal DNA had *nov*^{r} marker, the extent of separation of chromosomal DNA could be examined. Residual chromosomal DNA peaks two fractions faster than the plasmid DNA peak (figure 3). Our best estimate from biological data is that there is half a ng or less of chromosomal DNA contaminating the plasmid DNA in the peak fraction. By comparing with the intensity of the bands of serial dilutions of pBR 322 DNA of known concentrations, the pD7 concentration in fr. 7 was estimated as 1.4 µg/ml (and chromosomal DNA concentration from biological data was 0.25 ng/ml). Specific activity of the pD7 DNA is 170,000 cpm/µg of DNA; and 1 µg of DNA yields 2.7×10^7 *amp*^{r} transformants. Assuming a molecular weight of 12×10^6 for pD7 DNA, efficiency of transformation works out to between 5×10^{-4} and 1×10^{-3}.

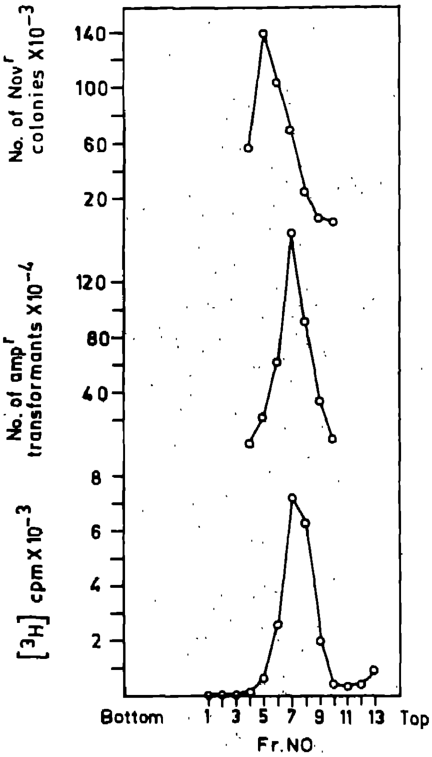

Figure 3. Radioactivity and biological activity profiles of $[^3H]$-labelled pD7 DNA and residual chromosomal DNA.

Biological activity of input intracellular pD7 DNA

Wild type and *rec* 1 cells of *H. influenzae* were transformed with $[^3H]$-labelled pD7 DNA (fr. 8; figure 3). Samples were taken out, washed and cleared-lysates prepared. From both the series, viz. wildtype or *rec 1* strain, the lysates were sedimented through sucrose gradients and DNA fractions were assayed for transformation on wild type cells. This provided information regarding the specific biological activity of intracellular input pD7 DNA. The data are recorded in table 4. The data indicate that: (i) substantial biological activity corresponding to reisolated pD7 DNA is recoverable, (ii) specific biological activity of pD7 reisolated from wild-type, assayed on wild type of later samples is lower than those of earlier samples suggesting inactivation events and (iii) in *rec* 1 strain, although the first transformation was low, the second transformation of reisolated DNA assayed on wild-type is reasonably high, suggesting that in *rec* 1 strains, pD7 is not permanantly damaged or blocked to a significant extent.

Table 4. Relative specific biological activity of gradient fractions.

Fraction No.	Recipient strain			
	Wild type		Rec_1^{--}	
	0 min	60 min	0 min	60 min
	No. of *amp*^{r} transformants/ [^{3}H]-cpm			
4	75	160	133	–
5	560	310	419	208
6	711	414	1142	206
7	731	386	869	225
8	1237	316	900	221
9	597	118	970	–
10	344	213	–	–

The lysates from cells treated for 0 and 60 min with pD7 DNA were sedimented through sucrose density gradients and the fractions were assayed for biological activity.

Discussion

Uptake of plasmid RSF 0885 DNA is stopped even when the chromosomal DNA is added some minutes after the mixing of competent cells with the plasmid DNA. Increased transformation with the recombinant plasmid DNA containing chromosomal DNA segments also suggests that plasmid DNA contains presumably only a degenerate version of the uptake sites required in *Haemophilus* (Sisco and Smith, 1979) which has a lower affinity for the receptor(s) in competent cells. In spite of the increase in transformation with plasmids carrying inserts, the efficiency of transformation is still less than 10^{-3}. Considering that close to 100% of the cells generally become competent in cultures grown in MIV competence media, the low transformation efficiency is presumably due to inactivation following uptake, at least a part of which was detected in the present experiments. Since the plasmid must be fixed intact in the cytoplasm, it would appear that any inactivation would interfere with its replication and fixation. On the other hand, fixation of the chromosomal DNA in *Haemophilus* should not be so sensitive to inactivation since a marker can be rescued by the resident chromosomal DNA even after inactivation occurs in the vicinity, but not in the marker. Inactivation in *rec* 1 strain is somewhat higher than in the wild type. Even so, it does not account for the much lower transofmation levels in the first transformation. Suprisingly, in *rec* 1, first transformation is low but the second one (lysate assay) is high. This suggests that up to 60 min, the block or damage introduced. in *rec* 1 transformation is not permanent. This block could be due to lack of *rec* 1 gene expression. It is, difficult to deduce if recombination *per se* is required for this or that *rec* 1 product acts in some other manner. Some amount of inactivation is observed both during transformation and transfection for the respective input DNAs and fragmentation has been noted (Voll and Goodgal, 1961; Notani and Goodgal, 1966; Notani, 1971; Notani *et al.*, 1973). Fragmentation for pD7 does not appear to be nearly as extensive as for example for transfecting DNA.

Quite unlike for OPM2 (Lacks, 1979), bulk of the intracellular input pD7 DNA is recoverable in biologically active from. This seems to be the genus-characteristic, since in *H. parainfluenzae* also, the heterospecific transforming DNA remains mostly double-stranded (Notani and Setlow, 1972).

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Microbial load in mass cultures of green algae *Scenedesmus acutus* and its processed powder

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MS received 3rd June 1981.

Abstract. Microbial contamination in cultures of the alga, *Scenedesmus acutus* raised in outdoor open tanks and also in the processed powder of the alga was monitored. The total bacterial population increased with time during the growth period of six days. When a combination of molasses and carbondioxide was employed as carbon source for this alga, the bacterial load increased to 10 colony forming units/ml. Yeast, molds and also coliforms were quantitated. Drum-drying the algae drastically reduced the bacterial load and storing the algal powder for a period of over 3 months did not increase the bacterial load. Pathogens like *Salmonella* and *Staphylococcus* were not detectable either in the open cultures or in the drum-dried algal powder. Although there are not set standards available in literature on the permissible level of the microbial contamination in algal biomass for use in foods, the microbial load appears to be within the limits of permissible levels stipulated by Indian Standard Institution standards for baby foods.

Keywords. Green alga; *Scenedesmus acutus*; microbial load; bacterial contaminants; processed powder.

Introduction

Algae are receiving wide attention as a source of biomass protein (BMP) for use in animal feeds and foods (Mituya *et al.*, 1953; Mateles and Tennenbaum, 1968; Clement *et al.*, 1969; Dabah, 1970; Soeder and Pabst, 1975; Becker and Venkataraman 1978; Becker *et al.*, 1976). The technology of algal cultivation and processing is now known for a few algae like *Chlorella, Scenedesmus, Coelastrum* and *Spirulina*. Clean water production of the green alga *Scenedesmus acutus* has been standardized at this Institute (Venkataraman *et al.* 1977a; Becker and Venkataraman, 1978). In this procedure, the alga is mass cultured in open cultivation basins and sterile conditions cannot be maintained. Under these conditions, the culture may be contaminated with heterotrophic bacteria even when it is grown autotrophically with simple inorganic media (Mituya *et al.*, 1953; Leone, 1963; Blasco, 1965; Vela and Guerra, 1966).

The possible health hazards of such contamination in mass algal cultures for animal feed and food cannot be overlooked. While extensive investigations have been carried out on the nutritional and toxicological safety of the algal biomass

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Abbreviations used: BMP, biomass protein; cfu, colony forming units.

(Soeder and Pabst, 1975; Becker *et al.*, 1976; Subbulakshmi *et al.*, 1976 Venkataraman *et al.*, 1977b and 1980), very little is known about the microbia contaminants. No reports are available on the bacterial load either in cultures o in processed material.

The common occurrence of different species of bacteria in algal cultures and i processed products raises the question of their significance in regard to publi health. Microbiological guidelines or standards for processed algal products ar needed to ensure the quality of the products and to protect consumers fror hazards.

The present study is an effort to determine the bacterial load and to quantitat and identify the organisms in algal cultures and processed algal powder. Th study was also intended to focus attention on the bacteriological aspects of alga biomass production.

Materials and methods

Cultivation of algae

Scenenedesmus acutus 273-3a was used in this study. Stock cultures wer maintained on agar slants. Inoculum for outdoor culturing was prepared fror slants in flourescent light illuminated thermostats with a light intensity of 5-7 K.lux Air was passed into the culture tubes at a rate of 300-500 ml/min. From the cultur tubes, the algae were transferred to glass carboys to build up enough inoculum fo mass culturing outdoors. The detailed procedure has been published elsewher (Venkataraman *et al.*, 1977a; Becker and Venkataraman, 1978). The alga cultures both indoors and outdoors were grown under non-aseptic conditions.

The algal cultures in outdoor tanks were concentrated by a centrifuge separato and dried on a drier (electrical or steam heated) or sundried on plastic sheets.

Growth Measurements

Algal growth and consequently the biomass produced are expressed as mg of alga produced/litre culture. This was computed by measuring absorbance of th cultures at 560 nm and converting the same into algal biomass with the help of standard graph. The normal growth period of *Scenedesmus* cultures when it i ready for harvest is about 6 days, and hence all measurements were taken for thi duration alone.

Storage

The dried algal biomass was stored in polyethylene-lined aluminium bags and kep for a period of three months for storage studies.

Sample preparation

For all the assays that are reported here, 10 ml of algal culture or 10 g processe algal powder were used. The material was mixed with 90 ml of 0.1% steril peptone water. This was shaken for 10 min and suitable dilutions were made fo the various assays.

Determination of viable count:

Bacteria: Standard methods were followed to determine the plate counts using nutrient agar media (Harringen and MacCance, 1976). Counts were expressed as colony forming units (cfu)/ml of culture or per g of algal powder.

Yeast and molds: These were assayed on acidified potato-dextrose medium. The plates inoculated with the samples were incubated at 25°C for 5 days followed by counting.

Coliforms: Coliform counts are expressed as most probable number using MacConkey broth (Harrigen and MacCance, 1976). Differential tests were used in the identification of the coliforms as *Escherichia coli, Enterobacter aerogens* and intermediate species (Speck, 1976).

Standard methods were used for determining the coagulase positive *Staphylococcus aureus* (Thatcher and Clerk, 1968). Suspected colonies were tested for coagulase activity. Analysis for *Salmonella* was carried out using the method of Thatcher and Clerk (1968).

Biochemical tests

The bacterial forms that were quantitated on the total plate counts were identified based on colony characteristics and biochemical tests as detailed in Bergey's manual (Breed *et al.,* 1957).

Results

Algal growth

As can be seen from table 1, the growth of alga with the addition of molasses alone was lower than with molasses and CO_2. The level of molasses used in the study appears to be optimal to prevent serious contamination by bacteria. This is evidenced by the absence of sugars in the culture medium when tested the following day after the addition of molasses.

Bacterial load

The bacterial load as a function of the age of cultures increased both in indoor and mass outdoor cultures. The increase was much less in indoor cultures during inoculum development as compared to the open basin algal cultures exposed completely to the environment. The bacterial load of cultures monitored at 0, 3 and 6 days of growth are summarized in table 1. The contamination is possibly from water and also from air. The excretion of certain organic substances by the algal cells into the media which may support bacterial growth has been reported in algal cultures that are grown in simple inorganic media (Vela and Guerra, 1966).

In indoor cultures, coliform group of organisms was not detected. In outdoor cultures, bacterial load increased from 0.3 to 45.0×10^5/ml within 6 days. However, there are some reports that the bacterial levels remain stable without significant increase in *Chlorella* cultures (Mituya *et al.*, 1953; Leone, 1963). Details of cultivation methods and the age of cultures at which the bacterial measurements were taken are not available for the above and hence this cannot be explained.

Table 1. Microbial load in *Scenedesmus acutus* cultures raised indoors and outdoors.

Conditions of cultures	Age of culture (days)	mg dry algae/ L	Total colony forming units/ml $\times 10^5$	Yeast & molds (colonies/ ml$\times 10^2$)	Coliform group (Most probable number/ml)
Indoor culture (Raised in thermostats)	Initial	60	—	—	Nil
	3	140	1.2 (1-5)[a]	1.0 (0.5-1.5)	Nil
	6	280	15 (8-25)	4-5 (3-9)	Nil
Mass outdoor cultures (Autotrophic culture 30L CO_2/h)	Initial	80	0.3 (0.2-4.0)	1.0 (0.3-2.0)	Nil
	3	200	18 (8-25)	5.0 (3-8)	29 (11-75)
	6	320	30 (25-60)	15 (7-25)	280 (210-460)
Heterotrophic culture (100 mg of sugarcane molasses/L)	Initial	80	0.3 (0.2-4.0)	1.0 (0.3-2.0)	Nil
	3	170	12 (5-20)	1.8 (1.0-6)	120 (75-210)
	6	260	45 (25-80)	5.0 (4-10)	180 (120-240)
Mixotrophic culture (30 L CO_2/h and 100 mg sugar-cane molasses/L)	Initial	80	0.3 (0.2-4)	1.5 (0.3-2)	Nil
	3	290	30 (20-80)	10 (3-25)	300 (240-460)
	6	480	400 (200-500)	30 (20-60)	900 (460-1100)

[a] Values in parenthesis indicate range

—Not detectable.

The values represent mean of five independent observations.

The coliform group of organisms was found only in a few batches. This type of contamination cannot be avoided under the pattern of cultivation used here. By suitable processing methods, they can be eliminated in the final product. Yeast and mold counts also increased in algal cultures during the growth period of six days.

Effect of organic carbon source

Sugarcane molasses is being considered as an alternative or supplementary carbon source to CO_2, which is rather expensive. Addition of sugarcane molasses even at a low level enhances the bacterial load in the cultures and this may be attributed to the presence of simple sugars in the medium. It is also possible that bacteria may oxidize the small amount of molasses rapidly and liberate CO_2 into the medium, thus stimulating algal growth.

Mixotrophic cultures have much higher bacterial load (4.0×10^7 cfu/6 days growth) compared to heterotrophic cultures. This needs serious consideration as the algal growth and consequently the biomass productivity is higher in mixotrophic cultures.

Pathogenic forms

The pathogenic forms like *Salmonella* and *Staphylococcus* were not found in the cultures at any stage of growth of algal cells.

The major contaminating organisms were identified by biochemical tests of one hundred randomly picked colonies. The results are listed in table 2. *Micrococcus* species were more common than those of Bacillus. Coliforms were much less frequent compared to other bacterial species. The various organisms of the coliform group identified are listed in table 3.

Table 2. Bacterial forms isolated from outdoor open *Scenedesmus acutus* cultures[a]

Organism	No. of isolates (%)
Micrococcus ureae	10
Micrococcus roseus	10
Micrococcus flavus	5
Micrococcus cryophilus	5
Micrococcus freundenreichii	8
Micrococcus varians	6
Micrococcus conglomeratus	10
Micrococcus caseolyticus	8
Bacillus subtilis	5
Bacillus coagulans	8
Bacillus macerans	3
Bacillus megaterium	2
Bacillus lentus	4
Bacillus brevis	2
Bacillus alvei	1
Bacillus circulans	4
Bacillus licheniformis	3
Unidentified forms	6

[a] Per cent occurrence of each bacterial species out of 100 randomly isolated colonies.

Table 3. Identification of coliform from mass outdoor cultures of *Scenedesmus acutus*[a]

Total number of isolates	Species of bacteria	Type I[b]	Type II[b]
100	*Escherichia coli*	20	5
	Intermediate	15	20
	Aerobacter	17	23

[a] Per cent distribution of the various types among one hundred randomly selected coliform colonies.

[b] Type I and II classified based on indole; methyl red; VP (Voges-Proskauer) and Citrate tests.

Effect of different methods of processing:

Sundrying, and drum-drying on electrical or steam heated driers have been used to prepare algal powder form the slurry. The microbial load in the dried powder is much lower than in cultures as would be expected (table 4). Drum-drying sterilizes the algal cells at least partially in spite of the short detention time (8-10 s). Drum-drying on electric dryer appears to reduce the microbial load and yeasts and molds more effectively than a steam-heated drier. This is probably due to lesser drum temperature being attained in the steam-heated drier.

Table 4. Microbial load in dried algal powder processed under different conditions[a]

Types of drying	Carbon source used in culture	Colonies forming units (c.f.u)/g ($\times 10^4$)	Yeasts and molds colonies/g ($\times 10^2$)
Electrically-heated drum drier	CO_2	2.0 (0.8-3.0)	1.0 (0.5-2.0)
Steam-heated drum drier	CO_2	3.0 (2.0-6.0)	6.0 (5.0-8.0)
Steam-heated drum drier	CO_2+ molasses	10.0 (5.0-15.0)	25.0 (10.0-30.0)
Sun dried	CO_2	120 (100-180)	140 (100-210)

[a] Products were free from *Staphylococcus, Coliform* group and *Salmonella.*

Values represent mean of five independent observations. Figures in parenthesis indicate the range of values.

When the cultures are grown mixotrophically with CO_2 and molasses, the microbial load is slightly higher than those grown with CO_2 alone. This may be due to the presence of spore-forming bacteria and also due to higher microbial load in the algal culture itself (table 1). The sun-dried algae had much higher microbial load than drum-dried material. No *Salmonella* and *Staphylococcus* were detected in the differently processed powders. Coliforms were totally absent in dried algal powder even though they were present in algal culture; possibly, they are destroyed during drying. The types of bacterial forms isolated from drum-dried algal material are listed in table 5. This differs significantly from the algal cultures in the total absence of the various species of *Micrococcus*.

Table 5. Bacterial forms isolated from drum-dried *Scenedesmus acutus* powder.[a]

Organism	No. of isolates (%)
Bacillus subtilis	20
Bacillus coagulans	25
Bacillus macerans	10
Bacillus alvei	5
Bacillus brevis	5
Bacillus lentus	10
Bacillus circulans	10
Unclassified	15

[a] Per cent occurrence of each bacterial species out of hundred randomly isolated colonies.

Storage studies

Data on storage studies conducted for a 3-month period using drum-dried algal powder are given in table 6. There was no increase in microbial load. This may be due to low moisture content (6-8%) which is not suitable for bacterial growth.

Table 6. Microbial counts on stored dried algal powder.[a]

Age of the powder (days)	Total plate counts Colonies/g $\times 10^4$	Yeast and moulds Colonies/g
Initial	9.0	600
15	7.0	550
30	7.0	550
45	6.5	500
60	6.0	450
90	5.5	400

[a] Values represent mean of three independent observations.

Temperature 28 ±2°C.

Discussion

Microbial monitoring of algal culture and algal powder is necessary, since it likely to affect the quality and safety of the final product. There are n internationally accepted safety limits that are available to draw a definite conclusion as to whether *Scenedesmus acutus* grown on a pilot plant scale at this Institut are within permissible limits.

However, the *Scenedesmus* grown in clean water and the drum-dried powde have microbial loads within probable safety limits, when compared to India Standards Institute (ISI) guidelines for baby foods which is 50,000 colonies/g. Th absence of pathogenic forms like *Salmonella* and *Staphylococcus* makes the alga powder safe for utilization. Drum-drying of *Scenedesmus* is an absolute necessit in order to rupture the undigestible cellulosic cell-wall for protein digestibility This evidently gives an effective sterilizing effect, reducing the microbial load Sun-drying is not a suitable method for processing *Scenedesmus* for the sam reason and this had been confirmed by earlier studies (Subbulakshmi *et al.*, 1976) Scenedesmus cultures are also contaminated by other organisms like protozoans other algal species and water insects and this aspect is not detailed here. However it may be mentioned that contamination of *Scenedesmus* by these is very limite and does not endanger either the culture or safety in its use as food.

The present world-wide emphasis has been more on technological and nutritiona aspects connected with algal biomass production. Though the possibility of usin algae as supplementary food protein has become less promising its use in feed is distinct possibility and the bacterial load is an important criterion for safety of use This should also receive more attention from the researchers in the field.

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Effect of protein malnutrition on the metabolism of uteral collagen in the rat*

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Abstract. Protein malnutrition in the rat resulted in a reduction in the syntehsis of collagen as determined by changes in the specific and total radioactivity incorporated following a single injection of [^{3}H]-proline. It was also accompanied by a retardation in the maturation of soluble to insoluble collagen. In addition, protein deficiency was accompanied by decreased rates of catabolism of both soluble and insoluble collagen.

Keywords. Collagen metabolism; uterus; protein-malnutrition.

Introduction

Protein malnutrition results in decreased tensile strength of wounds, tail tendons and skin in rats (Peacock, 1960) as well as impaired wound healing both in human and experimental animals (Edwards & Dumphy, 1958; Peacock, 1961; Stahl, 1963a; b). The results of a number of studies indicated that the level of protein in the diet had an effect on collagen metabolism. The collagen content and its synthesis were decreased in rats fed on a protein-deficient diet (Dickerson and John, 1969; Rao and Bose, 1969; Anasuya and Rao, 1970; McClain and Wiley, 1975; Angeleli *et al.*, 1978; Arumugam and Bose, 1979). Previous isotopic studies in this laboratory suggested a decrease in the metabolism of dermal collagen in protein deficient animals compared to controls (Rao and Rao, 1980a). The urinary excretion of hydroxyproline as well as hydroxylysyl glycosides postulated to reflect the turnover of body collagen was decreased in protein malnutrition indicating that the metabolism of collagen was adversely affected (Whitehead, 1965; Rao and Bose, 1969; Anasuya and Rao, 1970; Rao and Rao, 1980b).

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In the present investigation, incorporation of [^{3}H]-proline was used as an indicator of the synthesis as well as maturation of collagen and the catabolism of both the soluble and the insoluble collagen fractions in the uterus of normal and protein malnourished albino rats was studied.

Materials and methods

Twenty one day old weanling female albino rats were fed a basal diet for one week and then divided into two groups. The first group continued on the basal diet (control) and the second group was fed a low protein diet, described in table 1. The control rats were pair-fed with protein-deficient animals to compensate for the decreased food intake in the protein deficient animals (Rao and Bose, 1969). Water was freely available. Daily records of food consumption were kept and body weights were taken every week.

Table 1. Composition of different diets.

Constituents	Control diet % (gm)	Profein deficient diet % (gm)
Casein	20	6
Sucrose	30	30
Starch	39	53
Groundnut oil	6	6
Salt mixture*	4	4
Vitamin mixture**	1	1

* Salt mixture used had the following composition (in g) NaCl,105; KCl,120; KH_2PO_4,310; $Ca_3(PO_4)_2$,149; $CaCO_3$,210; $MnSO_4$ (anhydrous),0.20; $K_2Al_2(SO_4)_4$ 24 H_2O,0.09; $MnSO_4$ (anhydrous),90; $FePO_4.4H_2O$,14.7; $CuSO_4.5\,H_2O$,0.39; NaF, 0.57; KI,0.05. In addition the following three elements were also added (mg/kg diet) $ZnCl_2$,1.5 and $CoCl_2$ 6 H_2O,0.15.

** The vitamin mixture contained (mg/100 g diet) thiamine hydrochloride,0.8; riboflavin,0.8; pyridoxine hydrochloride,0.6; niacin,5; calcium pantothenate,4; inositol,20; choline chloride 200; folic acid 0.4, vitamin B_{12},2.0 μg; biotin,20 μg; vitamin A acetate,1000 I.U; vitamin D,150 I.U; α-tocoferol,12 mg and menadione,0.3 mg.

On the 23rd day, subgroups of control and protein-deficient animals were injected 30 μCi of [^{3}H]-proline per 100 g body weight, intraperitoneally [proline-T(G) sp. act. 1,500 mCi/mmol., obtained from BARC, Bombay]. Urine was collected under toluene for a period of 12 h after the administration of labelled proline. At intervals of 12 and 120 h, the animals were killed and uteri removed immediately for processing.

The remaining animals in both the groups received 50 μCi of [^{3}H]-proline per 100 g body weight intraperitoneally at the start of the experiment i.e. day 1. On day 28, urine was collected under toluene for a period of 24 h.

Analysis of uterus

The uteri were freed from the adhering tissues and the samples were homogenized. For the convenience of analysis, three uteri from each group were pooled together to make one sample thus making six samples in each group.

The minced material was extracted thrice with 0.45 M NaCl (pH 7.4) at 4°C for 24 h with one drop of octan-2-ol added as a preservative (Levene and Gross, 1959). An aliquot of the combined extracts containing the neutral salt-soluble collagen was hydrolyzed with an equal volume of 12 N HCl at 110°C for 24 h. The residue left after NaCl extraction, consisting of insoluble collagen was also similarly hydrolyzed. In the hydrolysates of both salt soluble and insoluble collagen, the hydroxyproline content, as well as the radioactivity of [^{3}H]-hydroxyproline was determined by the method of Rojkind and Gonzalez (1974). The collagen content was calculated by multiplying the hydroxyproline content by 7.46 (Neuman and Logan, 1950). The radioactivity was measured in an automated liquid scintillation spectrometer (Model LSS 34, Electronic Corporation of India Ltd., Hyderabad). The counting efficiency was 31% for tritium.

Analysis of urine

After hydrolyzing the peptides in the urine samples, the content and the radioactivity of hydroxyproline were determined as mentioned earlier.

Statistical methods

The results were statistically evaluated using Student's 't' test. The differences were regarded as significant at the level of $P<0.05$.

Results

The mean body weight of the control animals progressively increased from an initial value of 50 g to 92 g, after 28 days. The growth rate of the animals on the protein-deficient diet was found to be slower than that of control animals and reached a weight of 72 g after 28 days. The differences in the weight gain between the two groups are significant from the second week onwards and upto the end of the experiment.

Synthesis of collagen

To study the influence of protein malnutrition on the synthesis of collagen, the amount of incorporation of [^{3}H]-proline as [^{3}H]-hydroxyproline into the neutral salt-soluble collagen fraction of the uterus was examined at 12 and 120 h after the administration of labelled proline. This fraction, which contains the most recently synthesized collagen as well as some slightly older soluble collagen was found to be labelled maximally within 24 h after the injection of the isotope (Jackson and Bentley, 1960). The proportion of soluble collagen (calculated in relation to 100 mg of total collagen content in the uterus) in the protein-deficient animals was considerably lower than in the controls (table 2). The specific and total radioactivities of [^{3}H]-hydroxyproline in the protein-deficient animals at 12 and 120 h after the injection of [^{3}H]-proline were also significantly decreased as compared to controls (table 2).

Table 2. Effect of protein malnutrition on the yield of neutral salt-soluble collagen (NSC) and on the specific and total radioactivity of [^{3}H]-hydroxyproline into this fraction of uterus*.

Hours after [^{3}H]-proline administration		Neutral salt-soluble collagen		
		Content[a]	Specific radioactivity[b]	Total radioactivity[c]
12	Control	4.21±0.21	105.25±5.25	59361±4275
	Experimental	3.45±0.18**	82.5±4.25**	38115±2875**
120	Control	4.15±0.19	15.26±0.65	8485±625
	Experimental	3.27±0.16**	12.52±0.42**	5484±347**

* Values are mean ± SEM.
** Significant at $P<0.05$
[a] Expressed as mg/100mg collagen
[b] Expressed as dpm/ µg hydroxyproline
[c] Expressed as dpm/100 mg collagen

Conversion of soluble to insoluble collagen

Table 3 shows the yield of insoluble collagen in terms of the specific and total radioactivities of insoluble collagen at 12 and 120 h after the injection of labelled proline. Both the specific and total radioactivities of [^{3}H]-hydroxyproline in the insoluble collagen were decreased considerably in the protein-deficient animals as compared to controls.

Table 3. Effect of protein-malnutrition on the yield of insoluble collagen (INC) and on the specific activity of [^{3}H]-hydroxyproline in the INC of uterus*

Hours after [^{3}H]-proline administration		Insoluble collagen (INC)		
		Content[a]	Specific radioactivity[b]	Total radioactivity[c]
12	Control	95.79±0.21	1.52±0.11	19517±1237
	Experimental	96.55±0.18**	0.72±0.07**	9318±689**
120	Control	95.85±0.19	3.51±0.25	45096±2987
	Experimental	96.73±0.16**	1.42±0.12**	18412±1247**

* Values are mean ± SEM
** Significant at $P<0.05$
[a] Expressed as mg/100 mg collagen.
[b] Expressed as dpm/µg hydroxyproline
[c] Expressed as dpm/100 mg collagen.

When the total radioactivity of the soluble and the insoluble collagen was expressed as a percentage of the sum of both (table 4), 75% of the activity was present in the soluble collagen and 25% in the insoluble collagen in the control animals, 12 h after the injection of [^{3}H]-proline. The corresponding values for protein-deficient group were 80 and 20% respectively. After 120 h, 16% of the radioactivity was present in the soluble collagen and 84% in the insoluble collagen in controls, whereas the deficient group showed 23% in the soluble collagen and 77% in the insoluble collagen.

Table 4. Effect of protein malnutrition on the conversion of soluble collagen to insoluble collagen*

Hours after [^{3}H]-proline administration	Group	Total radioactivity of [^{3}H]-hydroxyproline in soluble collagen[a]	Total radioactivity of [^{3}H]-hydroxyproline in insoluble collagen[a]
12	Control	75	25
	Experimental	80	20
120	Control	16	84
	Experimental	23	77

* Data calculated from tables 2 and 3.

[a] The total activity of [^{3}H]-hydroxyproline in soluble and insoluble collagen is expressed in terms of percentage of the sum of both.

Catabolism of soluble collagen

The catabolism of soluble collagen was studied by measuring the urinary excretion of [^{3}H]-hydroxyproline during the first 12 h after [^{3}H]-proline injection. The urinary excretion of hydroxyproline and the specific radioactivity of [^{3}H]-hydroxyproline in the urine of protein-deficient animals were about two thirds of that in the controls (table 5). The total radioactivity of [^{3}H]-hydroxyproline was also decreased to a great extent (about 52%). Comparison of the total radioactivity of [^{3}H]-hydroxyproline in urine with that of the soluble fraction of collagen in the uterus of protein-deficient animals showed that the total radioactivity of [^{3}H]-hydroxyproline in urine (52%, table 5) was greater than in that of the soluble collagen (36%, table 2).

Table 5. Effect of protein-malnutrition on the urinary excretion of hydroxyproline and on the specific and total radioactivity of [^{3}H]-hydroxyproline during first 12 h after [^{3}H]-proline administration*.

Group	Urinary hydroxyproline		
	Excretion[a]	Specific radio activity[b]	Total radio-activity[c]
Control	165.4±11.25	27.55±1.95	4557±389
Experimental	108.5±8.37**	19.97±1.07**	2167±197**

* Values are mean ± SEM.

** Significant at $P<0.05$.

[a] Expressed as µg/12 h.

[b] Expressed as dpm/µg hydroxyproline.

[c] Expressed as dpm.

Catabolism of insoluble collagen

Urine was collected for 24 h on the 28th day after the administration of [^{3}H]-proline. The urinary excretion of hydroxyproline was about 56% of that in the controls (table 6). Eventhough both the groups exhibited similar specific radioactivity, the total activity of [^{3}H]-hydroxyproline in the deficient rats was only 70% of that in the controls.

Table 6. Effect of protein malnutrition on the urinary excretion of hydroxyproline and on the specific radioactivity and total radioactivity of [^{3}H]-hydroxyproline*

Days after [^{3}H]-proline administration		Urinary hydroxyproline		
		Excretion^{a}	Specific radioactivity^{b}	Total radio-activity^{c}
28	Control	375.27±21.25	1.47±0.11	551.65±41.27
	Experimental	246.52±12.22**	1.56±0.12***	384.57±21.25**

* Values are mean ± SEM.
** Significant at $P<0.05$
*** Not significant.

^{a} Expressed as μg/24 h.
^{b} Expressed ad dpm/μg hydroxyproline.
^{c} Expressed as dpm.

Discussion

Measurement of [^{3}H]-hydroxyproline after the injection of labelled proline i individual fractions of uterus collagen as well as its urinary excretion gives valuabl information on the metabolic turnover of collagen, its synthesis, the conversion o soluble to insoluble collagen and the catabolism of both soluble and insolubl fraction. The synthesis of collagen can be studied by following the radioactivity c [^{3}H]-hydroxyproline in soluble collagen within the first few hours and days after th injection of labelled proline. It has been reported (Prockop, 1964) that in youn rats, about two thirds of the urinary hydroxyproline is derived from the insolubl collagen and about one third from the soluble collagen fraction. It is therefor possible to study the catabolism of both soluble and insoluble collagen fractions b measuring urinary [^{3}H]-hydroxyproline 12 h and 4 weeks respectively, after th administration of labelled proline. The amount of [^{3}H]-hydroxyproline measure in soluble and insoluble collagen at 12 and 120 h yields information about th conversion of soluble to insoluble collagen.

Eventhough soluble collagen represents mainly newly synthesized collage (Jackson and Bentley, 1960), the degradation of insoluble collagen may als contribute to the amount of soluble collagen (Prockop and Kivirikko, 1968 Thus, the decrease in the content of soluble collagen in the uteri of protei deficient animals is in accordance with decreased collagen synthesis as well inhibited degradation of insoluble collagen.

In the present investigation, the specific activity of hydroxyproline in the solub collagen was decreased by about 18% while the total activity was more marked decreased (about 36%) due to the influence of protein malnutrition (table 2

These results therefore suggest that collagen synthesis is decreased in protein-deficient animals.

The lowered excretion of urinary hydroxyproline in protein-deficient animals (table 5 and 6) may be due to either decreased synthesis or decreased catabolism of collagen or both (Kivirikko, 1970). A diminished urinary excretion of hydroxyproline as well as of hydroxylysyl glycosides has also been reported in protein malnutrition (Whithead, 1965; Picou *et al.*, 1965; Widdowson and Whitehead, 1966; Anasuya and Rao, 1966; Rao and Bose, 1969; Anasuya and Rao, 1970; Rao and Rao, 1980b). The decreased total radioactivity of hydroxyproline in both uterine soluble collagen (table 2) and the urine (table 5) may be partly explained by decreased collagen synthesis. The total radioactivity of [^{3}H]-hydroxyproline was decreased more than the total radioactivity of [^{3}H]-hydroxyproline in the uterine soluble collagen. These results may therefore indicate decreased synthesis and catabolism of soluble collagen.

The results of the present investigation further suggest a decrease in the catabolism of insoluble collagen since the total radioactivity of urinary [^{3}H]-hydroxyproline measured 4 weeks after the administration of labelled proline was considerably lower in the protein-deficient animals.

The radioactivity of [^{3}H]-hydroxyproline in soluble and insoluble collagen measured at 12 and 120 h after the administration of labelled proline (table 2 and 3) also suggests an impaired conversion of soluble to mature, insoluble collagen in protein malnutrition.

These results therefore, collectively indicate that the synthesis of collagen, catabolism of both soluble and insoluble collagen and the maturation of uterine collagen are decreased in protein-deficient animals, compared to controls.

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Toxicity of carbaryl and 1-naphthol to four species of freshwater fish

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Abstract. The toxicity of the carbamate insecticide carbaryl (Seven®) and its metabolite, 1-naphthol, to four species of fish was studied. The calculated 96 h LC 50 values of carbaryl for *Catla catla* (Ham.), *Anabas testudineus* (Bloch), *Mystus cavasius* (Ham.) and *Mystus vittatus* (Bloch) are 6.4, 5.5, 4.6 and 2.4 ppm respectively and that of 1-naphthol are 4.3, 3, 0.33 and 1.1 ppm respectively. The degradation product of the insecticide was found to be more toxic than the parent compound, to all the four species studied.

Keywords. Pesticide toxicity; carbaryl; 1-naphthol; freshwater fish.

Introduction

Carbaryl (n-naphthyl, 1-methyl carbamate), is a carbamate pesticide that is being used widely to control a variety of pests of different crops. In India it is being extensively used against the pests of cotton, tobacco, paddy, vegetable crops and the like. It is considered to be environmentally safe because of its shorter persistence and lower mammalian toxicity (Brown, 1978) and hence is more acceptable than the 'environmentally hazardous' organochlorine and organophosphate pesticides. 1-Naphthol, the main degradation product of carbaryl, especially in the aquatic environment (Karimen *et al*, 1967), is supposed to be non-toxic to the nontarget organisms. However, the toxicity of 1-naphthol to the aquatic organisms does not seem to have been adequately tested and in the few studies on the toxicity to aquatic organisms of carbaryl and its degradation product the latter has been reported to be more toxic (Stewart *et al.*, 1967, Butler *et al.*, 1968 and Tilak *et al.*, 1980). Earlier, we reported that 1-naphthol was more toxic than the parent compound to two size groups of the carp, *Labeo rohita* (Ham.) (Tilak *et al.*, 1980).

The present investigation was undertaken to study the toxicity of carbaryl and 1-naphthol to the following economically important Indian fish: *Catla catla* (Ham.), *Anabas testudineus* (Bloch), *Mystus cavasius* (Ham.) and *M. vittatus* (Bloch).

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Materials and methods

The source of the fish, their size and average weight are shown in table 1. The fish were acclimatised to the laboratory conditions in well aerated, unchlorinated tap water at 28° ±2°C. During the period of acclimatization and experimentation, the fish were not fed. If the number of deaths exceeded 5% in any batch of fish during acclimatization, that batch was discarded. To test the toxicity of the two compounds, tests were conducted employing continuous flow systems, using large reservoirs and gradient flow as recommended in the report of the "Committee on methods of toxicity tests with aquatic organism (Anon, 1975)."

Table 1. Length, weight and source of fish used in the toxicity tests.

Species	Size range (cm)	Average wt. (g)	Source
Catla catla	1-2.5	0.3	Hatchery at Manchikalpudi, Guntur Dt., Andhra Pradesh[a]
Mystus vittatus	4.5-6	3.5	Guntur channel near Nambur, Andhra Pradesh
M. cavasius	5-6.5	4	—do—
Anabas testudineus	4-5.5	8	Kolleru lake, Andhra Pradesh

[a] The carps were being bred by injecting pituitary hormones.

Extraction of the pesticide containing water with methylene chloride at hourly intervals after the addition of the pesticide, followed by colorimetric analysis (Benson and Finnocchiaro, 1965) showed that for six hours following the addition of the pesticide, there was no detectable reduction in the concentration of the toxicant in the water in large glass reservoirs (24 to 30 L capacity). Hence, test solutions of desired concentrations were prepared in glass reservoirs, once in five hours and were let into the test containers through thin walled polyethylene tubes (vide recommendations of the above cited committee). The flow rate was adjusted with regulators so that four litres of water passed through the test containers in one hour. Standard solutions of the toxicants (100 mg/ml and 10 mg/ml) were prepared in acetone. The control fish received an equal quantity of acetone. The precautions suggested by the above cited committee in washing the containers, choice of the test fish, loading etc., were carefully followed. The length of the longest fish in any batch did not exceed one and a half times that of the smallest fish.

The hydrological conditions of the test medium are shown in table 2. The water used in the experiments was clear, unchlorinated ground water pumped from a deep well in the university campus.

Table 2. Chemical analysis of water used for toxicity tests.

Turbidity	8.5 silica units
Electrical conductivity at 28°C	816 micromhos/cm
pH	8.4
Total solids	680 mg/L
Total hardness	152 mg/L
Carbonate hardness as Ca CO_3	152 mg/L
noncorbonate hardness as Ca CO_3	nil
Alkalinity as Ca CO_3:	
Phenolphthalein alkalinity	10 mg/L
Methyl orange alkalinity	320 mg/L
Chloride as Cl^-	5.4 mg/L
Fluoride as F^-	2.6 mg/L
Iron	nil
Manganese	nil
Phosphate as PO_4^{3-}	nil
Sulphate as $BaSO_4$	148 mg/L
Nitrate as N	1 mg/L
Free chlorine	nil
Dissolved oxygen	8-10 ppm
O_2 absorbed in 4 hours for $KMnO_4$	0.6 mg/L

The sample of water is clear, colourless and odourless.

Different tissues of randomly selected control fish were extracted with methylene chloride. The extract was concentrated and analysed by both colourimetry and thin layer chromatography. The fish were found to be free from residues of carbaryl (detection limit, 0.1 ppm).

Pilot experiments were conducted to choose five test concentrations that resulted in mortality in the range of 10-90%. For each concentration, ten fish were tested and the experiment was repeated thrice. Finney's unweighted regression method of probit analysis (Finney 1971) as recommended by Roberts and Boyce (1972), was followed to calculate the 96-hour LC 50 values. The difference between the observed and calculated values was tested for significance using the Chi-square test.

Results

There were no deaths in the control fish during the period of experimentation. The 96-hour LC 50 values of carbaryl and 1-naphthol, with their 95% confidence limits are shown in table 3. The regression equations for the four species of fish whose tolerance to carbaryl and 1-naphthol was tested, are as follows: $Y=4.83X-8.25$ for carbaryl and $Y=6.61X-11.37$ for 1-naphthol for *A. testudineus*, $Y=2.6X-1.18$ for carbaryl and $Y=3.09X-1.34$ for 1-naphthol for *M. cavasius*, $Y=18.4X-46.6$ for carbaryl and $Y=25.36X-61.79$ for 1-naphthol for *C. catla*. The calculated LC 50 values of Carbaryl for *C. catla*, *A. testudineus*, *M. cavasius* and *M. vittatus* are 6.4, 5.5, 4.6 and 2.4 ppm respectively and of 1-naphthol, 4.3, 0.33 and 1.1 ppm respectively. Thus, for all the four species of fish, 1-naphthol was more toxic than the parent compound.

Table 3. Calculated 96-hour LC 50 values and their 95% confidence limits.

Species	Compound tested	LC 50 (ppm)	95% confidence limits	Calculated a^2 value
Catla catla	1-naphthol	4.3	4.2-4.4	4.03[a] (at 3 degrees of freedom)
	carbaryl	6.4	5-8.2	1.66[a] (at 4 degrees of freedom)
Mystus vittatus	1-naphthol	1.1	0.9-1.4	1.91[a] (at 3 d.f.)
	carbaryl	2.4	1.8-3.1	2.23[a] (at 3 d.f.)
M. cavasius	1-naphthol	0.33	0.25-0.4	0.36[a] (at 5 d.f.)
	carbaryl	4.6	4.1-5.2	5.52[a] (at 3 d.f.)
Anabas testutus	1-naphthol	3	2.7-3.4	2.65[a] (at 4 d.f.)
	carbaryl	5.5	4.8-6.3	1.91[a] (at 5 d.f.)

[a] not significant at 5% level. d.f., degrees of freedom.

Discussion

Although carbaryl and 1-naphthol were not as toxic to fish as many organochlorine and organophosphate pesticides, their toxicity was high enough to make them environmental hazards, if for any reason (rain immeditely after spraying, accidental spillage etc.) contamination of the aquatic environment should occur.

The four species of fish whose susceptibility to carbaryl and 1-naphthol was tested, belonged to three different families. It is surprising to note that the carp, *C. catla* which is supposed to be a very sensitive fish, incapable of withstanding environmental stress, is the least sensitive of the four, to both carbaryl and 1-naphthol. It may also be noted that in the case of the carp only fingerlings were used, whereas in the case of the other three adults were used. *Catla* of the same size as that of the other three species will have a higher LC 50 value (as it is well known that larger size groups have a higher tolerance to toxicants), in which case the disparity in the tolerance of the carp and the other species is very striking. A similar instance of greater sensitivity of air-breathing fish to endosulfan was reported by Rao (1979).

Between the two species of *Mystus,* carbaryl was relatively more toxic to *M. vittatus* than *M. cavasius,* whereas the reverse was true with 1-naphthol. Moreover, for *C. catla, A. testudineus* and *M. vittatus* 1-naphthol was more toic than carbaryl by a factor of one and half to two times, whereas for *M. vittatus* 1-naphthol was about 14 times more toxic than carbaryl.

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Dopaminergic mediation of γ-aminobutyric acid in the control of prolactin release: Plasma prolactin and brain tyrosine hydroxylase levels in ovariectomized conscious rats

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Abstract. The present experiments were carried out to further elucidate the mechanism by which dopamine mediates the actions of γ-aminobutyric acid on prolactin release from anterior pituitary following its intraventricular injection in overiectomized conscious rats. γ-Aminobutyric acid significantly suppressed the prolactin levels at 0.1 μmol concentration while at 4 μmol dose, the level was elevated. The activity of tyrosine hydroxylase was increased significantly in the anterior pituitary at the lower dose while the higher concentration of γ-aminobutyric acid did not bring about any change in the activity both in the hypothalamus and the anterior pituitary. The results presented suggest that intracellular dopamine in the anterior pituitary may directly inhibit prolactin release; the plasma prolactin level is elevated by γ-aminobutyric acid, by way of either inhibiting dopaminergic tone or possible stimulation of a physiological prolactin releasing hormone.

Keywords. γ-Aminobutyric acid; dopamine; prolactin; tyrosine hydroxylase.

Introduction

Although hypothalamic amines are known to participate directly or indirectly in he regulation of anterior pituitary hormones (MacLeod, 1976; Vijayan and McCann, 1978c, McCann *et al.*, 1979), the presence of dopamine the anterior ituitary (Saavedra *et al.*, 1975) suggests that intracellular adenohypophysial onoamines could also be involved in the regulation of the synthesis and/or elease of anterior pituitary hormones. The available evidence suggests that opamine released from the hypothalamic nerve terminals mediates the regulation f hormone release from the pituitary; in particular, dopamine exerts a tonic hibitory effect on the secretion of prolactin (Gibbs and Neill, 1978; Chiocchio *et* ., 1980). While some relationships have been found between gonadal hormones, ain biogenic amines and pituitary hormone release, the importance of γ-amino-tyric acid (GABA), an inhibitory neurotransmitter, as a possible mediator of terior pituitary hormone release is only now becoming evident (Ondo, 1974; ioduszewski *et al.*, 1976; Pass and Ondo, 1977; Rivier and Vale, 1977; Vijayan

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breviations used: GABA, γ-amino butyric acid; PRL, prolactin, dopamine, 3, 4-dihydroxyphenylethyl-ine; dopa. 3,4-dihydroxyphenylalanine.

and McCann, 1978a, b). Intraventricular microinjection of GABA elevated plasma lutienizing hormone and growth hormone and inhibited release of thyroxine stimulating hormone in overiectomized rats (Vijayan and McCann, 1978a; b). On the other hand, there was a dose related effect on the release of PRL which was decreased by low doses (Libertun and McCann, 1976; Vijayan and McCann, 1978a), and elevated by high doses of the transmitter (Vijayan and McCann, 1978a). Since GABA interacts with dopaminergic systems elsewhere in the brain (Giorguieff *et al.*, 1978; Negro-Vilar *et al.*, 1980), it is possible that dopamine might be an intermediate in the action of GABA on pituitary hormone release.

In the present work, we have evaluated the role of dopamine in the mediation of the action of GABA, following its intraventricular injection by correlating the changes in activity of tyrosine hydroxylase (EC 1.14.3a) the rate limiting enzyme in catecholamine synthesis, whose activity changes correlate well with alteration in dopamine levels (Saavedra *et al.*, 1974), with the effects on PRL release in ovariectomized conscious rats.

Materials and methods

Animals

Mature Wistar female rats weighing 180-200 g were housed under controlled conditions of light and temperature; they were fed on food pellets obtained from Hindustan Lever, Bombay, and had access to water *ad libitum*. The rats were ovariectomized after lightly anesthetizing with ether and used for experimentation 2-3 weeks later.

Experimental procedure

A 23 gauge stainless stell cannula (17 mm in length) was implanted into the 3rd ventricle of the experimental animals 5-7 days prior to the experiment as described previously (Antunes-Rodrigues and McCann, 1970; Vijayn and McCann, 1978c). A mandril prevented the obstruction of the cannula. The cannula was considered to be located in the 3rd ventricle if cerebrospinal fluid flowed continuously upon removal of the mandril. The animals were housed in individual cages.

γ-Aminobutyric acid (Sigma Chemical Co., St. Louis, Missouri, USA) was prepared freshly in 0.9% NaCl and 0.1 or 4 μmol dose in a volume of 2.5 μl was microinjected into the 3rd ventricle of unanesthetized rats using a 10 μl Hamilton syringe over a period of 60 s. These doses were chosen, since in earlier experiments, they were found to be effective in modifying plasma PRL levels (Vijayan and McCann, 1978 a, b; Negro- Vilar *et al.*, 1980). Controls received an equal volume of saline. Five or 15 min after the injection, the animals were killed by decapitation and the brains with the intact pituitaries were quickly removed and frozen immediately. Trunk blood was collected and plasma was separated by centrifugation at 4°C and stored frozen for assay of PRL. Anterior pituitaries and hypothalami (which include median eminence, medial basal hypthalamus and preoptic area) were dissected out according to the procedure described earlier (Vijayan, 1974) for the subsequent determination of tyrosine hydroxylase activity.

Tyrosine hydroxylase activity was assayed by the colourimetric method of

Shiman *et al.* (1971). The specific activity of tyrosine hydroxylase was expressed as nmol of 3, 4-dihydroxyphenylalanine (dopa) formed per mg of protein. Protein was estimated by the method of Lowry *et al.* (1951). Plasma PRL levels were measured with the radioimmuno assay kit supplied by NIAMDD, National Institutes of Health, Bethesda, Maryland, USA and results were expressed in terms of the RP-1 reference standard provided with the kit. All samples were run in one assay, each in duplicate to avoid interassay variation.

Results

Prolactin levels after intraventricular GABA administration

Plasma prolactin levels were relatively low in ovariectomized rats and were not altered by the intraventricular injection of saline. PRL levels were lowered slightly at 5 min by 0.1 μmol dose of GABA, whereas, the levels were significantly suppressed ($p<0.05$) at 15 min. On the other hand, the higher dose of 4 μmol produced an opposite effect in elevating prolactin levels significantly ($p<0.05$) at 15-min (figure 1).

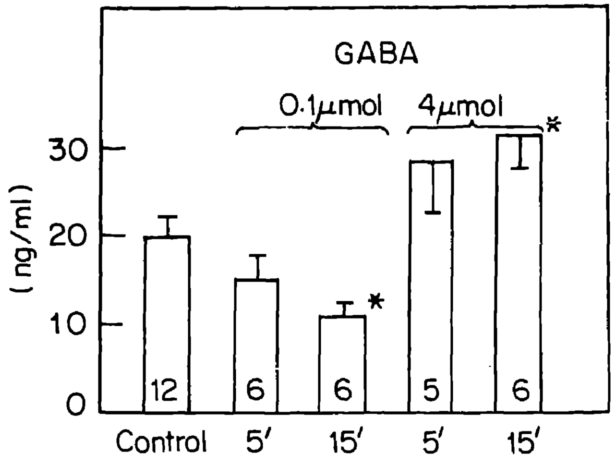

Figure 1. Changes in plasma prolactin (PRL) levels after intraventricular injection of GABA. In this and subsequent figures, numbers at the base of each column indicate the number of rats in each group; vertical lines above and below the mean represent the SEM.
* $P< 0.05$.

Tyrosine hydroxylase activity in the hypothalamus and anterior pituitary

Ovariectomy resulted in a marked increase in tyrosine hydroxylase activity in both the hypothalamus and anterior pituitary when compared to activity in these tissues of normal cycling rats (data not shown). Tyrosine hydroxylase activity in different areas of brain or anterior pituitary was not modified by the intraventricular injection of saline. There was no significant alteration of enzyme activity in the hypothalamus after administration of GABA either at 5 or 15 min, at the two dose levels (figure 2).

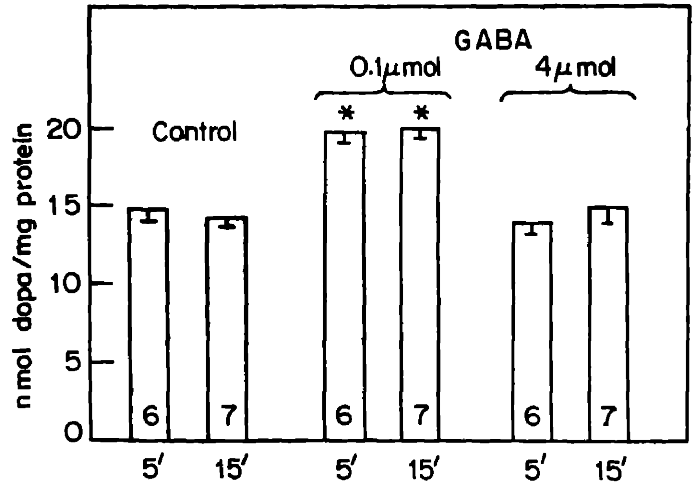

Figure 2. Changes in tyrosine hydroxylase activity of hypothalamus after intraventricular GABA administration.

Anterior pituitary tyrosine hydroxylase activity was significantly ($p<0.05$) increased with 0.1 μmol dose of GABA at 5 and 15 min while the higher dose of 4 μmol failed to modify it (figure 3).

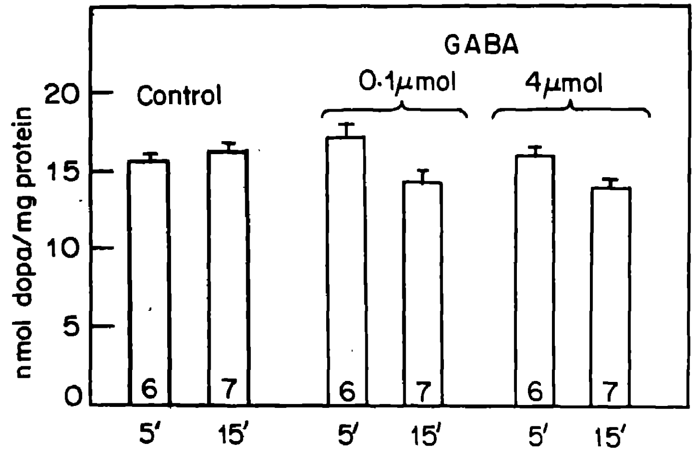

Figure 3. Changes in tyrosine hydroxylase activity of anterior pituitary after intraventricular GABA administration.

* $P<0.05$.

GABA did not produce any alteration in the enzyme activity of cerebral cortex which was taken as a tissue control.

Discussion

The present results indicate that conspicuous changes occur in the anterior pituitary catecholamine metabolism following intraventricular administration of 0.1 μmol of GABA, as evidenced by the significant increase in tyrosine hydroxylase activity. However, there was no clear evidence for an altered rate of dopamine synthesis in the hypothalmus, since there was no significant change in the enzyme activity in the hypothalamus with 0.1 μmol GABA. Furthermore, the higher dose of 4 μmol GABA also could not modify the rate of dopamine synthesis in the hypothalamus and/or anterior pituitary. GABA after its 3rd ventricular injection could possibly diffuse into the hypophyseal portal capillaries and be transported to the anterior pituitary where it stimulated dopamine synthesis. Negro-Vilar *et al.* (1980) recently reported a significant elevation in dopamine content of the median eminence following intraventricular administration of 0.1 μmol of GABA, while there was no change in dopamine levels of medial basal hypothalamus and preoptic area. This rise in dopamine in the median eminence was attributed to the synthesis of dopamine in the terminals of the tuberoinfundibular dopaminergic tract or to GABA induced release of dopamine from these terminals. The present results clearly indicate that the rise in dopamine in the median eminence noticed by Negro-Vilar *et al.*(1980) could possibly be due to the release of dopamine from the nerve terminals in median eminence since tyrosine hydroxylase activity in the hypothalamus was not modified. This apparently indicates the failure of GABA to induce *de novo* synthesis of dopamine in the hypothalamus. GABA agonists also have been found to alter dopamine turnover in the hypothalamus without altering the enzyme activity (Alderman and Shellenberger, 1974; Anden, 1974; Lakti and Losey, 1974).

The lower dose of 0.1 μmol GABA significantly suppressed plasma prolactin levels. Increased synthesis of dopamine by the anterior pituitary as evidenced by increase in tyrosine hydroxylase activity following the lower dose could directly inhibit PRL release from lactotrophs. Dopamine is known to have a tonic inhibitory action on PRL release (MacLeod and Lehemeyer, 1974). However, the higher dose of 4 μmol GABA significantly elevated plasma PRL levels, without altering the tyrosine hydroxylase activity. Receptors for both GABA and dopamine have been demonstrated in the anterior pituitary (Grandison and Guidotti, 1979; Kebabian and Calne, 1979) and the GABA agonist muscimol, has been shown to inhibit PRL release from the anterior pituitary at relatively low doses (Grandison and Guidotti, 1979). When dopaminergic receptors were blocked with pimozide, plasma PRL was lowered instead of elevated by higher doses of intraventricular GABA (Vijayan and McCann, 1979). The elevation in PRL by GABA could result from the inhibition of dopaminergic tone. The higher dose of GABA could inhibit prolactin inhibiting factor or stimulate the release of prolactin releasing factor that could stimulate PRL release. A recent report (Shin, 1980) suggests that the secretion of PRL during acute stress is due to the release of PRL releasing factor, and is not caused by the removal of an inhibitor. Acute ether stress in pimozide-pretreated rats consistently raised the levels of plasma PRL suggesting that the inhibitory factors are not involved, since their receptors were blocked and that a physiological releasing hormone must stimulate the release of prolactin.

In conclusion, our results strongly favour a role for intracelluar dopamine in the anterior pituitary as a mediator of GABA action in altering pituitary PRL release. It is also possible that GABA may have a direct action on the pituitary gland under physiological conditions.

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